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CONTRACT NO: DAMD17-90-C-0003

TITLE: Dietary Refinements in a Sensitive Fish Liver Tumor Model

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REPORT DATE: May 1, 1995

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1 May 95	Final 15 Nov 89 - 30 Sep 94

4. TITLE AND SUBTITLE
Dietary Refinements in a Sensitive Fish Liver Tumor Model

5. FUNDING NUMBERS
DAMD17-90-C-0003

6. AUTHOR(S)
David E. Hinton, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER
4B556

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

A purified diet has been developed and proven adequate for growth of the medaka (Oryzias latipes), a sensitive teleost species proven responsive to hepatocarcinogenic agents. The overall nutritional adequacy of the diet, a casein based ration, was evaluated and compared to three diets: commercially available flaked fish food; live newly hatched Artemia; and a combination of flake diet supplemented with Artemia. Survival, growth, reproductive success, general and liver histopathology, and selected hepatic enzyme activities were compared in medaka from first feeding through reproductive maturity. The PC-diet proved adequate in all of the above criteria. This diet provides a standardized, nutritionally adequate and consistent alternative to undefined conventional diets and is less likely to contain the range of xenobiotics possible in whole live food. Studies in progress indicate the suitability of the diet for aquatic toxicity/carcinogenicity studies.

14. SUBJECT TERMS
Teleost, liver, carcinogenesis, medaka, Oryzias latipes, model diet, fish

15. NUMBER OF PAGES
243

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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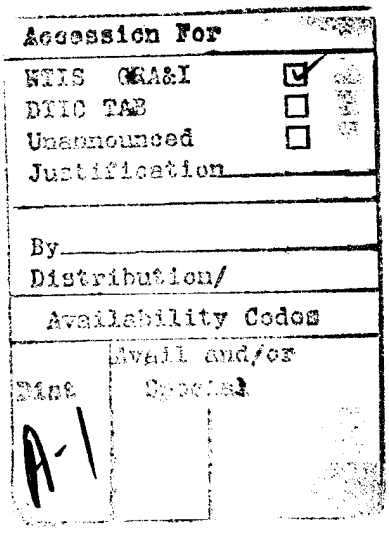

David E. Hinton 4/24/95

PI - Signature **Date**

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5. INTRODUCTION

The medaka (*Oryzias latipes*) has been used extensively to investigate experimental embryology in biology laboratories (Kirchen and West, 1976; Yamamoto and Egami, 1974). Within the last two decades, work performed by Japanese investigators (Aoki and Matsudaira, 1977; Aoki and Matsudaira, 1981; Aoki and Matsudaira, 1984; Aoki and Matsudaira, 1986; Egami and Etoh, 1969; Egami, et al., 1981; Ishikawa, et al., 1984; Ishikawa, et al., 1975; Ishikawa and Takayama, 1979; Kyono, 1978; Kyono and Egami, 1977; Kyono, et al., 1979; Kyono-Hamaguchi, 1984; Masahito, et al., 1989; Masahito, et al., 1988; Matsushima and Sugimura, 1976; Takayama and Ishikawa, 1977) and subsequently by U.S. investigators (Hawkins, et al., 1991; Hawkins, et al., 1986; Hawkins, et al., 1985a; Hawkins, et al., 1988a; Hawkins, et al., 1988b; Hawkins, et al., 1985b; Hawkins, et al., 1990; Hawkins, et al., 1988c; Hinton, 1989a; Hinton, In Press; Hinton, et al., 1988b; Hinton, et al., 1985; Hinton, et al., 1984a; Hinton, et al., 1988a; Hinton, et al., 1992a; Klaunig, et al., 1984; Laurén, et al., 1990) have shown that this fish species has great promise as a vertebrate model for toxicity including carcinogenicity. The time to tumor when the animal is exposed to diethylnitrosamine or to methylazoxymethanol acetate is brief. The latency period for hepatocarcinogenesis with these compounds is frequently 8 - 16 weeks. In addition to the response to carcinogens in the laboratory, this fish with its small size, ease of rearing, and other economic advantages (Laurén, et al., 1990; Matsushima and Sugimura, 1976) is being used to investigate toxicity and carcinogenicity of extracts from sediments at various polluted harbors and estuaries (Fabacher, et al., 1991).

Although the medaka (*Oryzias latipes*) shows great promise as a model for carcinogenesis

(Aoki and Matsudaira, 1977; Egami, et al., 1981; Ishikawa, et al., 1975; Ishikawa and Takayama, 1979), its potential is currently limited by the lack of a defined diet. Refinements in detection of early preneoplastic lesions and improved definition of windows of bioavailability consistent with sensitivity in developing host are needed.

Current, closed-formula, commercial rations are derived largely from fish meal and plankton of unknown source and variability and may contribute confounding environmental contaminants. Since these diets are flaked, attempts to add test compounds for the purpose of conducting chronic dietary exposures have been made more difficult. The result is that we are limited to studies with so-called complete carcinogens (Aoki and Matsudaira, 1977; Egami, et al., 1981; Ishikawa, et al., 1975; Ishikawa and Takayama, 1979). It is generally agreed that the number of environmentally relevant chemicals which modulate (promote or inhibit) carcinogenesis exceeds the number of initiators of the process. Studies of modulation of carcinogenesis require a model which consistently provides an expected tumor incidence with a given dose of initiating carcinogen. Furthermore, since dietary contaminants are important modulators of carcinogenesis, a highly purified, defined diet to which test agents may be added is essential.

We seek to develop an open-formula defined diet (OFD) for use in carcinogenesis studies with medaka. Advantages of using medaka as *in vivo* screens in cancer bioassay have been reviewed (Consensus Committee, 1984; Hatanaka, et al., 1982; Matsushima and Sugimura, 1976). Although a partially defined diet has been available (Sinnhuber, et al., 1977) and used to investigate modulation of carcinogenesis in the rainbow trout (*Oncorhynchus mykiss*) model (Nixon, 1984), environmental characteristics of medaka require use of a diet more suitable for

warm water species. In contrast to the larger trout, amount of total diet required for medaka makes use of a defined ration cost effective.

The NCI and US EPA Consensus Committee (1984) recommended use of defined rations so that attempts to analyze modulatory effects of environmentally relevant chemicals on carcinogenesis in this promising model would be possible. The UC Davis Aquatic Toxicology Laboratory with expertise in anatomy, pathology, physiology, biochemistry, toxicology and nutrition of fishes, has undertaken research to refine elements of this promising vertebrate model for carcinogenesis.

The development of new, faster eukaryotic models for bioassay to identify potential carcinogens and modulators of carcinogenesis is a pressing need for research. As is stated above, medaka develop tumors of liver after exposure to proven mammalian kenotoxic carcinogens. Their brief latency periods, sensitivity to a variety of procarcinogens, and decreased cost while maintaining large number of exposed individuals argue for further development of this model. However, the lack of a defined diet and absence of established tumor incidence data under standardized and reproducible conditions are current limitations for development.

The overall goal of the research was to develop a sensitive fish model for studying carcinogenic potential and modulatory effects of environmental agents on carcinogenesis. To reach this goal, we have identified four enabling objectives: 1) produce a nutritionally adequate, open-formula purified diet for medaka; 2) determine levels of xenobiotic metabolizing enzymes in medaka fed this and a conventional diet; 3) establish incidences of liver tumors in medaka fed the open-formula diet; and 4) compare the frequency of liver tumor formation in carcinogen-initiated medaka fed the open-formula diet versus those fed a conventional dietary

regime. All objectives have been achieved. What follows is a full report on a nutritionally adequate, open-formula purified diet for medaka and the growth and overall health characteristics of fish fed this versus a conventional regime (DeKoven, 1990) (Chapter 1). In Chapter 2, we present our results from a study comparing frequency of liver tumor formation in medaka briefly exposed to diethylnitrosamine, a hepatic carcinogen in fishes and fed either the open-formula purified ration or a conventional dietary regimen. In chapter 3 we present our results from a study comparing frequency of liver tumor formation in medaka administered a six-month dietary exposure to aflatoxin B_1 (AFB_1) and fed either the open-formula purified ration or a conventional dietary regimen.

6. BODY

Chapter 1

Purified Diet for medaka (*Oryzias latipes*): Refining a Fish Model for Toxicological Research

Introduction:

Small aquarium fishes are receiving increasing attention as vertebrate test organisms for rapid *in vivo* screening of suspect carcinogens and as monitors of environmental pollution in aquatic field surveys (Hoover, 1984; Vogelbein, et al., 1990). One of the most promising small fish species is the medaka orange-red or golden variety) (Aoki and Matsudaira, 1977; Bunton, 1990; Egami, et al., 1981; Hinton, et al., 1988b; Hinton, et al., 1984a; Kyono and Egami, 1977; Laurén, et al., 1990). Medaka are tolerant to a wide range of environmental conditions, and their small size and rapid maturation supports uncomplicated breeding and maintenance of large numbers in the laboratory. Aspects of the physiology, development and genetics of this species are known (Briggs and Egami, 1959; Yamamoto, 1975). Medaka have proven to be particularly sensitive to a variety of carcinogens (Hatanaka, et al., 1982), forming tumors after relatively short (3 - 4 months) induction periods (Hawkins, et al., 1988b; Masahito, et al., 1989). In addition, individual medaka exhibit hepatic enzyme responses to polyaromatic hydrocarbon (PAH) xenobiotics similar to other vertebrate models (Schell, et al., 1987).

Given the potential importance of medaka as a vertebrate model, the need exists for a nutritionally adequate and consistent experimental diet for this species (Bailey, et al., 1984). Conventional medaka diets, such as *Artemia* nauplii and/or closed-formula, commercially

available flake diets, are not ideal for most carcinogenesis or toxicity bioassays because of the risk of additional contaminant exposure through the diet which may alter the toxic response of the fish (Consensus Committee, 1984). Live foods, such as *Artemia* nauplii, can be a source of adventitious xenobiotics (Olney, et al., 1980; Seidel, et al., 1982). Additionally, different geographical strains of *Artemia* cysts may contain varying amounts and types of chlorinated and aromatic hydrocarbons and heavy metals (Olney, et al., 1980; Seidel, et al., 1982). Although adventitious contaminants in non-purified commercial fish feeds have not been evaluated, changes in liver ultrastructure, including accumulation of lipid droplets and proliferation of rough endoplasmic reticulum, have been reported in fish fed non-purified, "practical" feed ingredients common to commercially available diets (Affandi and Biagianti, 1987; Bac, et al., 1983; Mosconi-Bac, 1987). Additionally, the incidence of liver tumors, after exposure to identical concentrations of aflatoxin B_1, was significantly higher in rainbow trout (*Oncorhynchus mykiss*) fed a diet based on fish protein concentrate compared to trout fed purified casein-based diets (Lee, et al., 1978).

While nutritional adequacy of conventional diets for medaka has yet to be rigorously evaluated, decreased growth and reproduction have been noted when commonly used flake feeds were fed alone (Hirshfield, 1980; Stanley, 1977). Although *Artemia* nauplii generally support good growth and survival, their nutrient composition may vary depending on geographical strain or season during which the cysts were collected (Cowgill, et al., 1987; Fujita, et al., 1980; Klein-Macphee, et al., 1980; Schauer, et al., 1980; Seidel, et al., 1982). Such variations significantly affect growth and survival of both fish and crustacean larvae (Amat, et al., 1987; Beck, et al., 1980; Johns, et al., 1980; Klein-Macphee, et al., 1980; Klein-Macphee, et al., 1982;

Seidel, et al., 1982).

Neither the effect of diet nor the nutritional status of medaka have been considered when evaluating metabolic or tumorigenic responses to experimental toxicants. Several studies with small mammals and other fish species, however, indicate that the nutritional status of the experimental animals and nutritional variation between different experimental diets may influence metabolic response and sensitivity to xenobiotics (Andersson, et al., 1985; Ankley and Blazer, 1988; Hickie and Dixon, 1987; Mehrle, et al., 1974; Mehrle, et al., 1977; Sachan, 1975; Stott and Sinnhuber, 1987; Wade, et al., 1985).

A standardized nutritionally adequate purified diet, suitable for maintaining medaka through all life stages, would overcome many of the problems associated with conventional diets (Bailey, et al., 1984). A purified diet would be more nutritionally consistent and less likely to contribute xenobiotics. Purified diets have been successfully developed for rainbow trout and used in toxicity bioassays and long-term feeding studies (Halver, 1957; Halver and Coates, 1957; Hendricks, 1982; Yu, et al., 1979); however, these diets were not formulated for warm water species such as the medaka. To date, purified diets have not been used to rear medaka from first feeding through reproductive maturity.

A purified casein-based diet (PC-diet), was formulated (DeKoven, 1990) by modifying a purified ration developed by Conklin *et al.* (1980). Two dietary trials were undertaken to evaluate the nutritional adequacy of PC-diet compared to conventional feeding regimes presently used in other laboratories (Aoki and Matsudaira, 1977; Bunton, 1990; Egami, et al., 1981; Schell, et al., 1987). Medaka growth, survival, reproductive success including embryo performance, general histology, and activities of selected hepatic enzymes were evaluated and compared.

Materials & Methods:

Diets: Both formulated and live diets were used. Trial 1 compared the nutritional adequacy of the purified casein-based diet (designated as PC-diet) to a live food (A-diet) and a closed formula, commercially available flaked diet for tropical fish (FL-diet). The A-diet consisted of newly hatched brine shrimp (*Artemia* spp.) nauplii (San Francisco Bay Brand, Newark, CA), while the FL-diet consisted of a conventionally used commercial flake diet, Kordon Stress Flakes (Kordon Co., Hayward, CA). In Trial 2, medaka were fed either the PC-diet or a combined regime of a commercial flake diet (Tetramin, Tetrawerke, Germany[1]) plus brine shrimp nauplii (designated as F/A-diet). The composition of the PC-diet is given in Table 1-1 and proximate analyses (Jones 1984) of the PC-, FL- and A-diets are shown in Table 1-2.

The formulated diets were prepared in 100 g (Trial 1) or 1 kg (Trial 2) batches and stored at -20°C. Purified ingredients were obtained from U.S. Biochemical Corporation (Cleveland, OH) and ICN Nutritional Biochemicals (Cleveland, OH). Dry ingredients for the PC-diets were mixed with a rotary mixer for 15 minutes. The oils were mixed with the tert-butyl hydroquinone (TBHQ; Aldrich Chemicals, Milwaukee, Wisconsin) and mixed well into the dry ingredients. Distilled water was slowly added to form a slightly cohesive mixture. In Trial 1, the PC-diet was then pressed through a nylon sieve (1 mm mesh size) and dried at 50°C for 15 minutes. In Trial 2, the diet was pressed through a stainless steel sieve (1.4 mm mesh size, U.S. Standard Tyler Sieve, #14) and freeze dried at - 80°C in a Labconco freeze dryer.

In both trials, fish were fed to slight excess twice daily and tanks were siphoned daily to remove uneaten food and feces. The F/A-diet group was fed flaked food five days per week and brine shrimp nauplii two days per week. In both trials, particle sizes of the formulated diets were

increased as the fish grew. From hatch to 4 weeks, the fish were fed formulated diets ranging from 100 to 250 µm particle diameter. After 4 weeks, the fish were fed particle sizes ranging from 250-µm to 850-µm particle diameter. Newly hatched brine shrimp nauplii (A-diet and F/A-diet) were separated from unhatched and empty cysts and rinsed with distilled water before being fed to the fish.

System design: Golden variety medaka were reared in a static system (Trial 1: larvae and juveniles) or in a recirculating aquarium system (Trial 1: adults; Trial 2: all life stages) (Nunez and Hinton In Prep.). Water temperature was maintained at 25 ± 0.5°C. Ammonia and nitrite levels were monitored daily (Trial 1) or weekly (Trial 2) and maintained at $\leq$ 0.1 ppm. Nitrates, pH, conductivity and hardness were monitored weekly in Trial 2. Dissolved O_2 was maintained at or near saturation.

Experimental Methods: Medaka eggs were collected from broodstock maintained at 25°C under a 16L:8D photoperiod. Eggs for all experiments were pooled from several females and incubated in aerated modified (no methylene blue) embryo rearing media (Kirchen and West 1976; Rugh 1962) at 25 ± 1°C. The embryo rearing media was replaced daily at which time dead embryos were removed. Larvae hatched 9 to 10 days after the eggs were collected.

In both trials, initial mean wet and dry weights and morphometric parameters were determined by sampling 30 newly hatched unfed normal larvae. The larvae were killed with an overdose of tricaine methane sulfonate ("Finquel", Argent Co., Redmond WA). Standard lengths (length from the tip of the snout to the end of the vertebral column) were measured in Trial 1 using an ocular micrometer and a dissecting microscope. Morphometric analyses of total length, maximum depth and maximum width were measured in Trial 2 using a computer-assisted image

analysis system (Nikon Micro-plan II, Laboratory Computer Systems Inc., Cambridge, MA) and a dissecting microscope. Euthanized larvae were then carefully wicked dry, placed in pre-weighed foil cups and weighed on an ultramicrobalance to 0.00001 g (Trial 1) or 0.0001 g (Trial 2). The larvae were dried at 60°C for 24 h, cooled in a desiccator and then weighed.

Trial 1

Newly hatched larvae with normal swim bladder inflation were randomly sorted and equally distributed among nine 1.5-L glass jars, 20 fish per jar. Each diet treatment (PC, A and FL) consisted of three replicates, randomly assigned to the nine jars. All fish in each jar were measured and wet weights were determined at 7, 9 and 12 weeks. Food was withheld for 24 hours prior to sampling. Immediately before sampling, the fish were lightly anesthetized with tricaine methane sulfonate (50 ppm), and standard lengths were measured as described above. Each fish was then carefully wicked dry, transferred to a tared, covered 5-mL beaker of reconstituted water, weighed on a microbalance (to 0.01 mg), and then placed in jars filled with 1.5 L of aerated, reconstituted water to recover. Mortality associated with the above procedure was low (1.6 - 3.0 %) during the 12 weeks. Instantaneous growth rates (IGRs) (Weatherley and Gill 1987), based on natural log (Ln) of wet weight measurements, were determined for the replicates of each diet treatment from 0 to 7, 7 to 9 and 9 to 12 weeks by the formula:

$$\frac{(\text{Ln wet weight final} - \text{Ln wet weight initial})}{\text{\# days}} \times 100$$

Surviving fish at 12 weeks were used to establish adult growth, survival, and reproductive success of broodstock under different diet treatments. The triplicate groups of fish from the respective diet treatments were pooled in 37-L acrylic aquaria in a recirculating system such that all aquaria received the same reconstituted water and were subjected to the same temperature and

light regimes. The time to first egg production for females from each diet treatment was recorded.

At week 24, 18 fish from each treatment were randomly sampled for wet weight determinations. Of these, 15 individuals from each treatment were dried at 60°C for 24 h and dry weights determined. The remaining three fish were fixed in Davidson's solution, embedded in resin and stained with toluidine blue for histological evaluation. Additionally, 12 females and six males from the PC- and A-diet treatments were randomly selected and sorted into three broodstock groups (henceforth called "broodstock"), each consisting of 4 females and two males. The broodstock were housed in mesh-bottomed PVC cylinders in a recirculating system. The six broodstock containers were suspended in a random arrangement (see Appendix 1) in two 100-L tanks, and identical rearing conditions were maintained.

Evaluation of broodstock reproductive success was conducted during weeks 29, 31 and 34. Eggs were collected over 3 days from each replicate broodstock group and incubated separately. Percent viable hatch was determined for replicates from both sample periods. Eggs collected during week 34 were evaluated for developmental abnormalities. Abnormal embryos were removed and incubated separately until they hatched or died. Hatchlings were evaluated for normal development; i.e., swim bladder inflation and normal swimming behavior (Marty, et al., 1990).

Trial 2

Newly hatched larvae with normal swim bladder inflation were pooled and equally distributed among ten 37-L acrylic aquaria, 250 larvae per aquarium. Diet treatments (PC and F/A) were randomly assigned to aquaria with 5 replicates per diet.

Ten fish per replicate were sampled from different regions in each aquarium every 10 days for wet and dry weight determinations as described for Trial 1. At 30, 60, 90 and 110 days, morphometric analyses of total length, maximum depth, and maximum width were conducted. Instantaneous growth rates were determined for wet and dry weights and for morphometric parameters (Weatherley and Gill, 1987). Samples for general histology were taken at 20 day intervals. Fish were anesthetized, placed in Karnovsky's fixative, and processed routinely in paraffin for hematoxylin and eosin (H & E) staining.

At days 35, 70, and 110, 20 fish from each diet treatment were randomly selected for analysis of enzyme activity: Ethoxycoumarin 0-deethylase (ECOD), Glutathione S-transferase (GST), and Gamma-glutamyl transferase (GGT). Visceral masses (day 35 only) or livers (day 70 and 110) were dissected free from anesthetized fish, pooled according to diet treatment, and frozen at -80°C. Samples were homogenized in cold sucrose buffer with a Potter/Elvehjem (Teflon/glass) homogenizer. The S9 fraction was prepared by centrifugation of the homogenate for 30 min at 9000 x g. Ethoxycoumarin 0-deethylase, a mixed function oxidase enzyme, was estimated by the production of 7-hydroxycoumarin. The supernatant was assayed fluorimetrically for ECOD activity as described (Greenlee and Poland, 1978). Glutathione S-transferase, a microsomal and cytosolic conjugating enzyme, was measured spectro-photometrically using chloro-dinitrobenzene (CDNB) as the substrate (Laurén, et al., 1989). Gamma-glutamyl transferase (GGT), a cytosolic phase II enzyme found primarily in the kidney, was assayed using Sigma Kit 545-1 (Sigma Chemical Co., St. Louis, MO). Protein was quantitated by the method of Bradford (1976).

Fish were monitored daily and the time to first egg production and the number of eggs

produced by each replicate of each treatment were recorded. Reproductively active fish were removed from the treatment aquaria to serve as broodstock. These fish were placed in separate 37-L aquaria and maintained on their respective diet treatments. After acclimatization, eggs were collected from these broodstock over two periods of 5 days. Eggs obtained from the broodstock of each treatment group were incubated separately. and evaluated for developmental abnormalities and viable hatch as described in Trial 1.

Statistical Analysis: Data were analyzed according to statistical methods described by Sokal and Rohlf (1981). All data presented as percent values were arc-sin transformed before analysis. In Trial 1, standard lengths, wet weights, and instantaneous growth rates of the larvae and juveniles were analyzed by one-way nested ANOVA. Differences were ranked by the Scheffe test. Percent hatch and survival were compared by the Student's t-test and one-way ANOVA, respectively. Data from Trial 2 were evaluated using the Student's t-test. A significance level of 0.05% was used throughout the study. Enzymatic data from Trial 2 were evaluated using the Spearman's rank correlation. A significance level of 0.5 was used for this nonparametric test.

Results:

Fish readily consumed all diets in both diet trials. Larvae began feeding approximately 24 to 48 hours after hatching, as noted by the presence of food particles in the gut. Medaka fed at the water surface, as noted by Yamamoto (1975), as well as at the bottom and in the water column.

Trial 1

Nutritional adequacy of the different diets was evaluated in terms of growth, survival, histology and reproductive success of medaka.

Survival and growth: Survival to 12 weeks was not significantly different in any of the diet treatments. Percent survival (mean ± SE) was as follows: PC-diet = 83.3 ± 4.0%; A-diet = 91.7 ± 7.7%; FL-diet = 80.0 ± 9.8%). Most deaths occurred within three weeks of hatching and these fish were notably smaller than their cohorts. Survival from 12 to 24 weeks was similar in both the PC- and A-diet treatments (80% and 83.3%, respectively). Survival of fish fed the FL-diet was much lower during this period (48.3%), and 76% of the surviving fish (22 of 29) were pale and emaciated. Necropsies of moribund and dead fish from the FL-diet group revealed depleted muscle tissue and fat deposits.

Significant differences in wet weights and standard lengths were noted between fish reared on the different diet treatments. Fish fed the FL-diet had significantly lower wet weights and standard lengths at 7, 9, and 12 weeks than fish fed either the PC- or A-diets (Figure 1-1). Average standard lengths of the fish fed PC- and A- diets did not differ significantly at 7, 9, or 12 weeks; however, corresponding wet weights of fish fed the A-diet were significantly greater. There were no significant differences in length of fish fed the PC-or A-diets (Figure 1-1). At 24 weeks, fish reared on the FL-diet had significantly lower wet and dry weights. Wet and dry weights were not significantly different between adults reared on the PC- or A-diets (Table 1-3).

Mean rates of change (i.e. growth) between sample periods were evaluated using IGRs. Wet weight IGRs of all diet treatment groups were highest in the period from hatch to seven weeks and decreased over time (Table 1-4). This decrease in IGRs was significant in all three diet treatments. Fish fed the PC- and A-diets did not have significantly different wet weight IGRs from hatch to 7 weeks, however, IGRs of fish fed the FL-diet were significantly lower during this period. Wet weight IGRs were not significantly different between any of the diet

from 7 to 9 weeks or from 9 to 12 weeks.

Histology: Histological evaluation revealed no abnormalities in the internal organs of the fish reared on the PC- or A-diets; however, there was extensive muscle wasting in fish fed the FL-diet. Muscle bundles of these fish were atrophic and there were large spaces between adjacent muscle bundles. No other abnormalities were noted in the internal organs of the fish reared on the FL-diet.

Skeletal deformities: No spinal deformities were noted in medaka fed the PC- or A-diets, but one female in the FL treatment group had kyphosis.

Reproductive success: Medaka fed the A-diet were the first to become reproductively active with the first eggs appearing at 13 weeks posthatch. Females fed the PC-diet first produced eggs at 14 weeks. By 24 weeks, all of the females in the PC- and A-diet groups had been observed with eggs. Broodstock fed the A-diet produced eggs with a slight orange tint, whereas PC eggs were uncolored. Only 2 out of the remaining 29 FL-fed fish produced eggs (noted at 23 and 24 weeks). Due to the lack of reproductively active females from the FL-diet group, these fish were not used for further experiments evaluating reproductive success.

The number of eggs collected per female for evaluation of reproductive success ranged from 22 to 80 for PC-diet broodstock and 28 to 60 for A-diet broodstock. Percent viable hatch did not differ significantly between diet groups (PC-diet: 94.4 ± 1.2%; A-diet: 94.4 ± 1.3%). Incidence of developmental abnormalities was low and did not differ significantly between diet treatments (PC-diet: 5.6 ± 2.4%; A-diet: 5.6 ± 1.9%).

Trial 2

Survival and growth: Survival to 110 days was not significantly different in either of the

diet treatments (PC-diet 95.0 ± 0.5%; F/A-diet 96.7 ± 0.5%). Fish fed the F/A-diet had significantly greater mean wet weight, total length, and maximum depth at all sample points after day 0 (Figure 1-2; Table 1-5). The highest IGRs, calculated from wet weight, occurred during the interval between hatch and 30 days for both diet treatments. Instantaneous growth rates of the F/A treatment, however, were significantly higher than those of the PC treatment during this period (Figure 1-3). Between 30 and 40 days, IGRs were significantly higher in fish fed the PC-diet. After 40 days, IGRs were not significantly different between diet treatments, except between 90 to 100 days.

Analyses based on morphometric parameters of total length, maximum depth, and maximum width (Table 1-5) showed fish fed the F/A-diet had significantly greater IGRs for all morphometric parameters for the interval of 0 to 30 days (Figure 1-4). However, between 30 and 60 days the PC-diet fed fish showed significantly greater IGRs for total length, maximum width and total depth. Maximum depth and maximum width IGRs were also significantly greater in the PC treatment group from 60 to 90 days. There was no significant difference in IGRs between diet treatments after 60 days (total length) or 90 days (maximum width and depth and total length).

Liver Histology: No differences in liver lipid or glycogen were seen between the F/A-diet and the PC-diet. In both diet groups, we observed occasional, pale, rounded hepatocytes with brightly eosinophilic cytoplasmic bodies and nuclear eosinophilia with marginated chromatin. These degenerating and necrotic hepatocytes, classified as apoptotic cells, appeared in similar numbers in F/A fed fish (3 of 51 livers) and PC-diet fed fish (3 of 49 livers). Apoptosis occurred as early as 40 days posthatch and continued sporadically through the end of the study (110 days

posthatch).

Liver Biochemical Parameters: The ECOD and GST activities for the two feeding regimes were similar except for on day 110 where GST activity appeared higher (significant to 95% level; Spearman Rank correlation) in fish fed the PC-diet (Table 1-6). Both the F/A- and PC-diet groups at 35 days had detectable levels of GGT (12.45 and 12.74 nmol/min/mg protein respectively). Activity of GGT was not detectable at 70 or 110 days in fish fed either diet.

Skeletal Deformities: Skeletal deformities were found in 67 adult fish from the F/A-fed groups (average from all aquaria = 5.4%). Gross lesions ranged from slight axial deformation to multiple lateral (scoliosis) and dorsoventral (lordosis/kyphosis) curvatures (Figure 1-5), as well as mild cranial abnormalities. The dorsoventral curvature, while difficult to ascertain grossly, seemed to occur between the second and third vertebrae. Radiographically and histologically, spinal deformities were more obvious. The F/A-diet-related vertebral abnormalities were less severe than the congenital "wavy tail" abnormality (not observed in this study) that has been previously described (Yamamoto, 1963). Only one fish with a very slight axial deformation was found in the PC-diet fed group during the final count (0.08% incidence).

Reproductive success: Time to first egg production was not significantly different between diet treatments (mean F/A-diet= 92 ± 3.8 days; mean PC-diet= 90 ± 2.2 days). There were no significant differences between the total number of eggs (PC-diet= 156 ± 19.2 ; F/A-diet= 144 ± 48.35) or the numbers of fertilized (PC-diet= 141 ± 19.1; F/A-diet= 122 ± 43.8) and unfertilized eggs (PC-diet= 15 ± 1.2; F/A-diet= 22 ± 4.9), produced from the onset of egg production to 110 days in either diet treatment. At 110 days, however, the proportion of fish which did not show external sexual differentiation was significantly higher in the PC-diet group

(34.1 ± 2.0%) compared to the F/A-diet group (15.5 ± 1.1%). Percent viable hatch was similar in both diet treatment groups (pooled mean PC-diet = 85.1%; pooled mean F/A-diet = 83.7%), and incidences of developmental abnormalities were low (pooled mean PC-diet = 0.95%; pooled mean F/A-diet = 0.90 %) for offspring of adults.

Discussion

Despite the use of different rearing systems in Trials 1 and 2, results of this study have demonstrated the overall nutritional adequacy of the PC-diet. Adequate growth and development, with no deleterious effects, resulted when medaka were fed with a regime comprised solely of the purified diet. The PC-diet meets the recommendations of the Consensus Committee (1984). Its use provides a way to decrease experimental variables by helping to define the dietary needs of medaka while obviating the use of live food. The PC-diet is suitable for maintaining medaka from first feeding to reproductive maturity, and provides a standardized, nutritionally adequate and consistent alternative to conventional diets.

Potential long-term problems may result when medaka are fed a conventional diet (FL- or F/A-diets). The incidence of skeletal deformities in the F/A group (Trial 2) appears to be diet related, because such alterations were not observed in the PC-diet fed group. It is unlikely that skeletal deformities were the result of water-borne contaminants (Couch, et al., 1977) or aquarium/system factors because medaka in both diet treatments were reared under identical conditions in the same recirculated water. Genetic factors were not likely to be responsible for skeletal alterations in this study because medaka were randomly selected from identical broodstock. A heritable recessive trait known as "wavy tail" exists in medaka (Yamamoto, 1963); however, this external characteristic, apparent at hatch, was not seen in larvae initially

selected in our study.

The FL-diet alone does not appear to meet the nutritional requirements of medaka. Poor growth, reduced survival, and emaciation were noted solely in fish fed the FL-diet (Trial 1) and reproductive success was low in this diet treatment. The connection between poor growth and survival, and nutritionally inadequate or imbalanced feeding regimes is well documented (Roberts and Bullock, 1989), whereas the influence of nutritional status and reproductive success may vary between species (Blaxter, 1988). In medaka, however, inadequate broodstock nutrition may compromise fecundity or maternal somatic growth (Hirshfield, 1980).

Reproductive success (i.e., fecundity and egg hatchability) and larval viability did not differ significantly between medaka fed the PC-diet and fish fed A-diet (Trial 1) or F/A-diet (Trial 2). Additionally, in Trial 2, growth rates (as IGRs) of PC-diet broodstock were comparable to, or higher than, broodstock fed the F/A-diet after day 30 . These results indicate that the nutritional status of PC-diet broodstock was not compromised such that somatic growth was sacrificed for egg production.

Coloration was the only difference between eggs of broodstock fed the diets containing live food (A- and F/A-diets: orange eggs) and those from medaka fed the PC-diet (colorless eggs). Egg pigmentation is derived from (Craik, 1985; Harris, 1984; Mommsen and Walsh, 1988; Schaeffer, et al., 1988) and appears to be proportional to levels of carotenoids in the maternal diet (Harris, 1984). Takeuchi (1960) showed that female medaka fed a carotenoid-free diet produced colorless eggs. The PC-diet, without carotenoids, produced similar results.

Our results indicate that reproductive success or subsequent embryo and larval viability in medaka are not affected by an absence of carotenoids in the PC-diet. The role of carotenoids

in reproductive success and embryo viability of fish is speculative, remaining unsubstantiated by controlled laboratory experiments (Choubert, 1986; Tacon, 1981; Watanabe, et al., 1984). While studies show egg yolk carotenoids are a source of chromatophore pigment in newly hatched larvae (Mommsen and Walsh, 1988; Steven, 1949), absence of chromatophore pigments apparently does not affect larval survival or viability. Torrissen (1984) found no effect of egg carotenoid level on embryo and alevin survival in Atlantic salmon. Similarly, Harris (1984) and Dabrowski et al. (1987) were unable to correlate broodstock reproductive success with dietary carotenoid.

Liver histological and biochemical changes were observed during growth in juvenile fish. Apoptosis, seen in both the F/A and PC treatments, is a transient alteration that may reflect tissue remodeling (Wyllie, et al., 1980) as part of generational growth-related phenomena. Livers of medaka from both diet treatments showed detectable levels of ECOD and GST activity. This indicates that both diets provide adequate nutrition for development of the xenobiotic metabolizing enzymes necessary for detoxification and activation of endogenous and foreign compounds. Both ECOD, a good indicator of the constitutive level of cytochrome P-450 monooxygenase activity, and GST, a conjugating enzyme, increased from day 75 to day 110. Increases in P-450 and Phase 2 conjugating enzymes have been documented to increase during development in rodents (Zongzhu, et al., 1982) but this is the first citing of this increase in medaka. This is important because changes in metabolism with age of the animal may affect its response to toxicants (Zongzhu, et al., 1982). The presence of GGT activity at day 35 but no later, may represent remnants of GGT isozymes, and in this way, be analogous to rodents where activity is fetal and neonatal only (Fiala, et al., 1972). Alternatively, livers at day 35 were so

small that dissection was very difficult and assays were conducted on visceral masses. Visceral masses frequently contain kidney which is generally rich in GGT at all life stages (Fiala, et al., 1972). On day 110 post-hatch, higher GST levels in PC-fed fish were observed. Glutathione S-transferase is used in protection from toxic injury (Kaplowitz, 1980) and the higher levels of GST in PC-fed fish may reflect the enhanced nutritional status of these fish. It is unlikely a water-borne effect, since the same recirculated water was used with both diet treatment groups.

Although the present formulation of the PC-diet appears to be nutritionally adequate, the significant lag in early growth rates (ie. 0-30 days), and resultant differences in weight and morphometric parameters need to be addressed. Superior growth of fish fed live food versus formulated diets has been well documented in other species and seems especially apparent during the larval stage (Appelbaum, 1985; Dabrowski, et al., 1984; Dabrowski and Kaushik, 1985; Dabrowski and Poczyczynski, 1988a; Dabrowski and Poczyczynski, 1988b; Jobling, 1986; Lauff and Hofer, 1984). Several hypotheses have been proposed including: (i) preference of first-feeding larvae for live and motile food compared to inert food (Appelbaum, 1985); (ii) auto-digestion of previously live food in the gut, resulting in breakdown of complex nutrients to simpler molecules more readily assimilated by larval fish, which may lack fully developed digestive systems (Dabrowski, et al., 1984; Dabrowski and Kaushik, 1985; Dabrowski and Poczyczynski, 1988a; Dabrowski and Poczyczynski, 1988b; Lauff and Hofer, 1984); and (iii) overload of the digestive and absorptive capacity of the larval gut when fed (comparatively) high energy, formulated feeds (Jobling, 1986).

Leaching of water-soluble nutrients from the PC-diet prior to consumption may be a factor contributing to lower initial growth rates. Cloudiness of the water immediately surrounding diet

particles was noted after the PC-diet was first added. This could indicate leaching which would lower the quality of food or promote bacterial overgrowth. Poor visual acuity and prey capture efficiency by larval medaka may add to this problem. Although medaka larvae are actively free-swimming and able to capture prey within 24 hours of hatching, their visual acuity is not fully developed until approximately 3 weeks of age (Ohki and Aoki, 1985).

Although the delay in initial growth of medaka fed the PC-diet was reflected in lower wet and dry weights (Trials 1 and 2), the fish were able to compensate for these differences. In Trial 1, there was no significant difference in wet or dry weights at 24 weeks, and we have subsequently found that Trial 2 fish maintained on either diet treatment to 35 weeks posthatch did not differ significantly in wet or dry weights. This "recovery growth" pattern exhibited by medaka fed the PC-diet is similar to that noted for rainbow trout fingerlings starved and then fed *ad libitum* (Weatherley and Gill, 1981). In the rainbow trout, as in medaka, there were no deleterious effects of this early lag in growth. For medaka reared on the PC-diet, development to maturity, as well as hatchability or viability of offspring were not compromised.

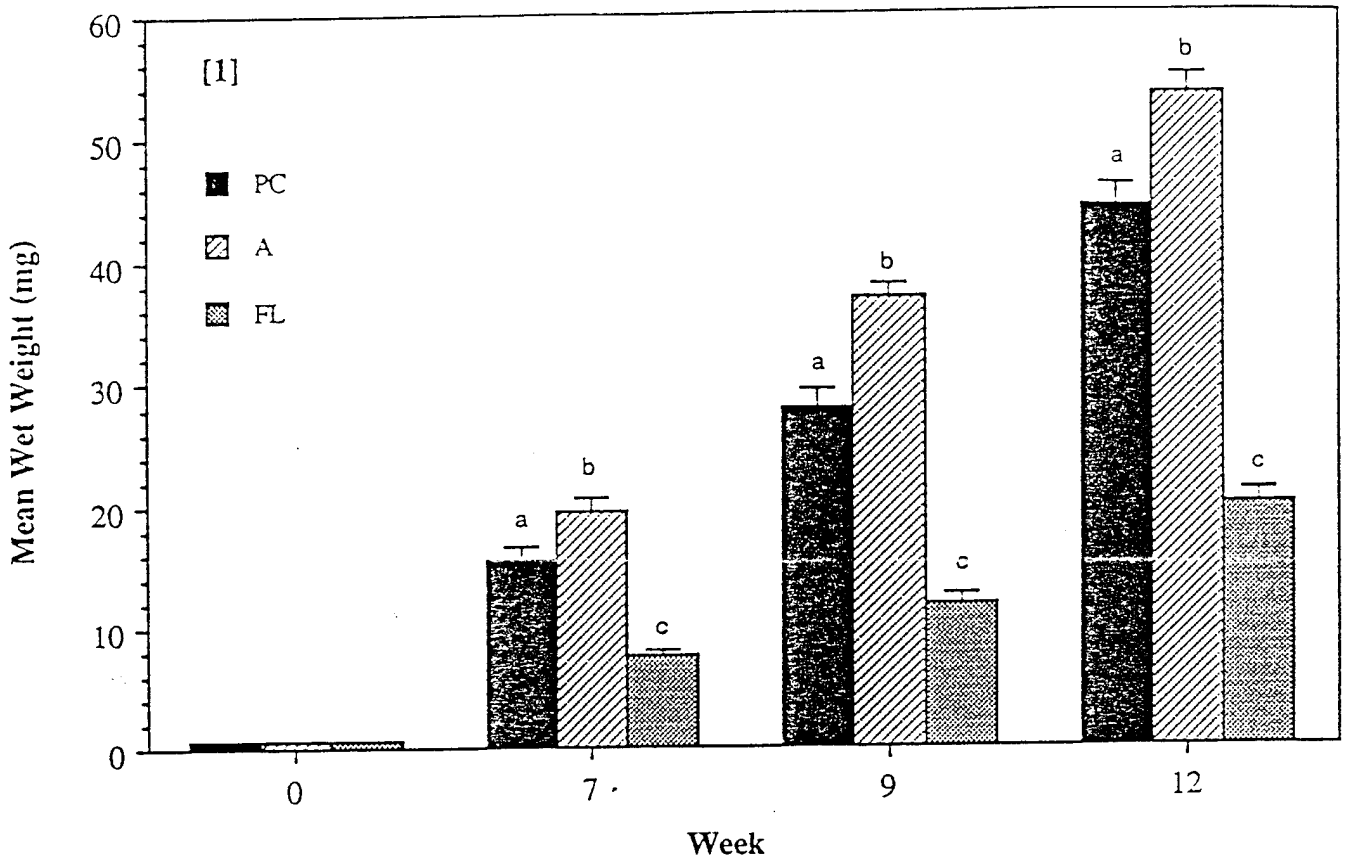
The undefined lipid portion (soy lecithin, corn and cod liver oil), of the PC-diet remains an area of concern. Adventitious dietary xenobiotics, which may modulate toxicity (Leatherland and Sonstegard, 1982; Leatherland, et al., 1979; Malins, et al., 1988; McCain, et al., 1988), may be introduced with this component. Initial attempts to replace the lipid portion of the PC-diet with purified fatty acids were not successful (DeKoven, 1990); however, we continue to pursue this aspect of medaka nutrition.

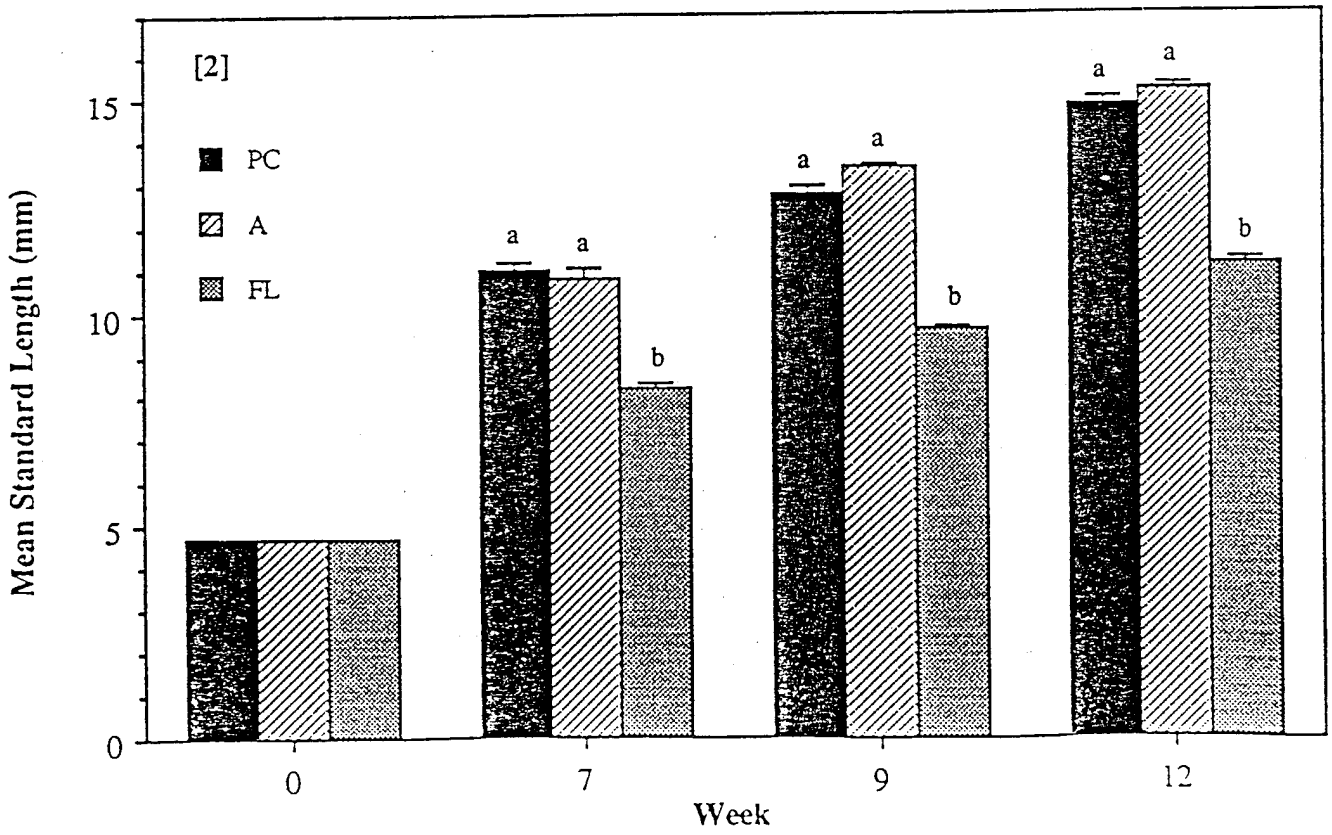
Although further refinements are needed in the PC-diet, it has distinct advantages over conventional diets. It is more nutritionally consistent and does not require separate culture of

food items; hence, it is much less labor intensive than feeding live foods. It is also less likely to contain the range of xenobiotics possible in whole, live food. As a standardized, purified diet, the PC-diet would decrease variation in results both between testing laboratories and in serial studies within a given laboratory. The PC-diet is also a good vehicle for delivering known amounts of experimental toxicants, a complex and questionable ambition using live food. Finally, because of the purified, open formulation of the PC-diet, it can be used to evaluate dietary modulation of toxicity through manipulation of amounts and types of selected nutrients. This opens a new area of research with medaka, one which is not possible with live foods or commercial closed-formula diets presently in use.

LEGENDS FOR FIGURES

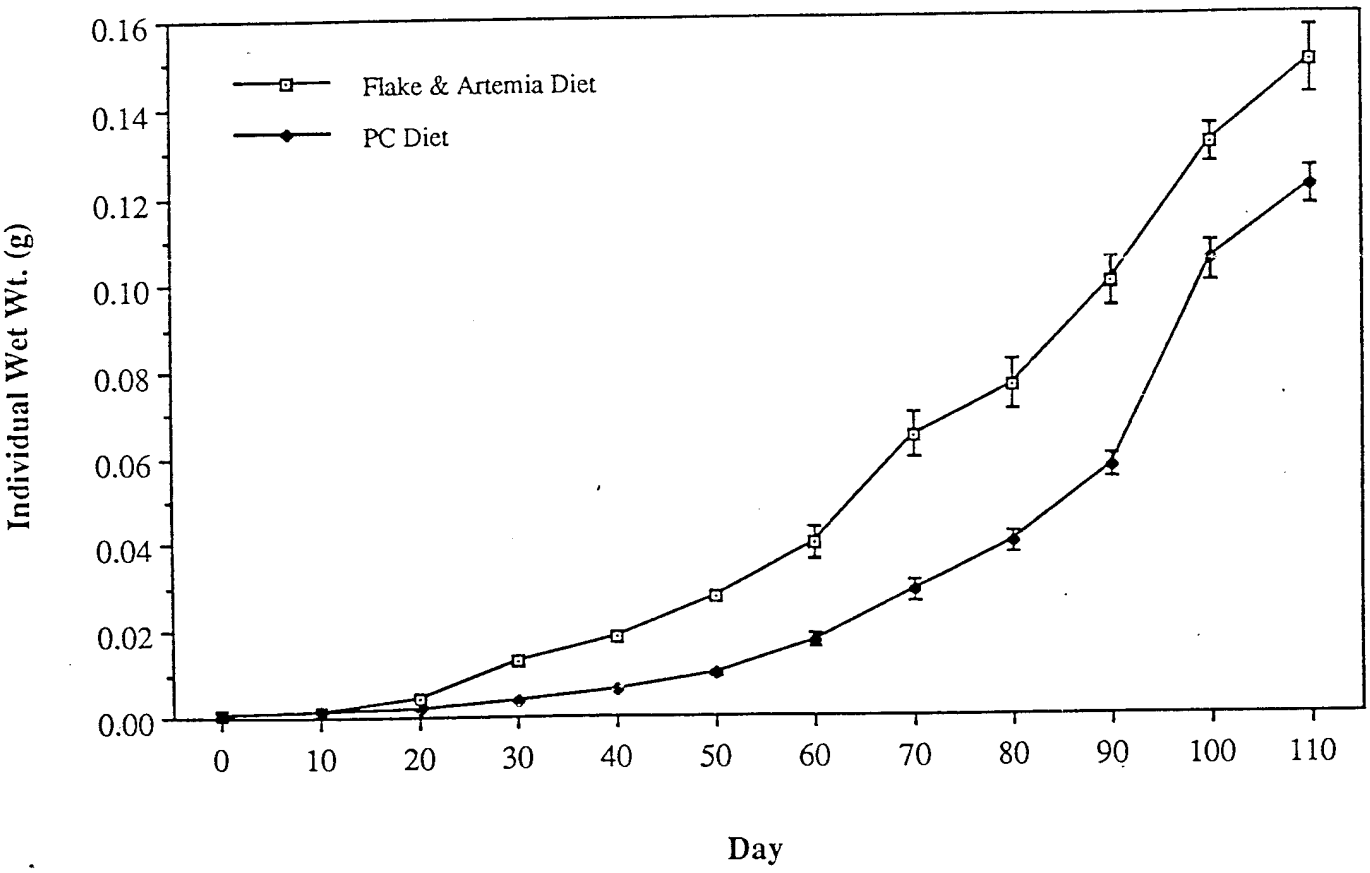
Figure 1-1. Trial 1. Average wet weight [1] and average standard length [2] ($\pm$ SE) of medaka reared on Flake (FL), Purified Casein (PC) and live *Artemia* (A) diets. Bars within same sample period showing different letters are significantly different $P \leq 0.05$.


[1]
PC
A
FL
Mean Wet Weight (mg)
60
50
40
30
20
10
0
a
b
c
a
b
c
a
b
c
0
7
9
12
Week


[2]
PC
A
FL
Mean Standard Length (mm)
15
10
5
0
a
a
b
a
a
b
a
a
b
0
7
9
12
Week

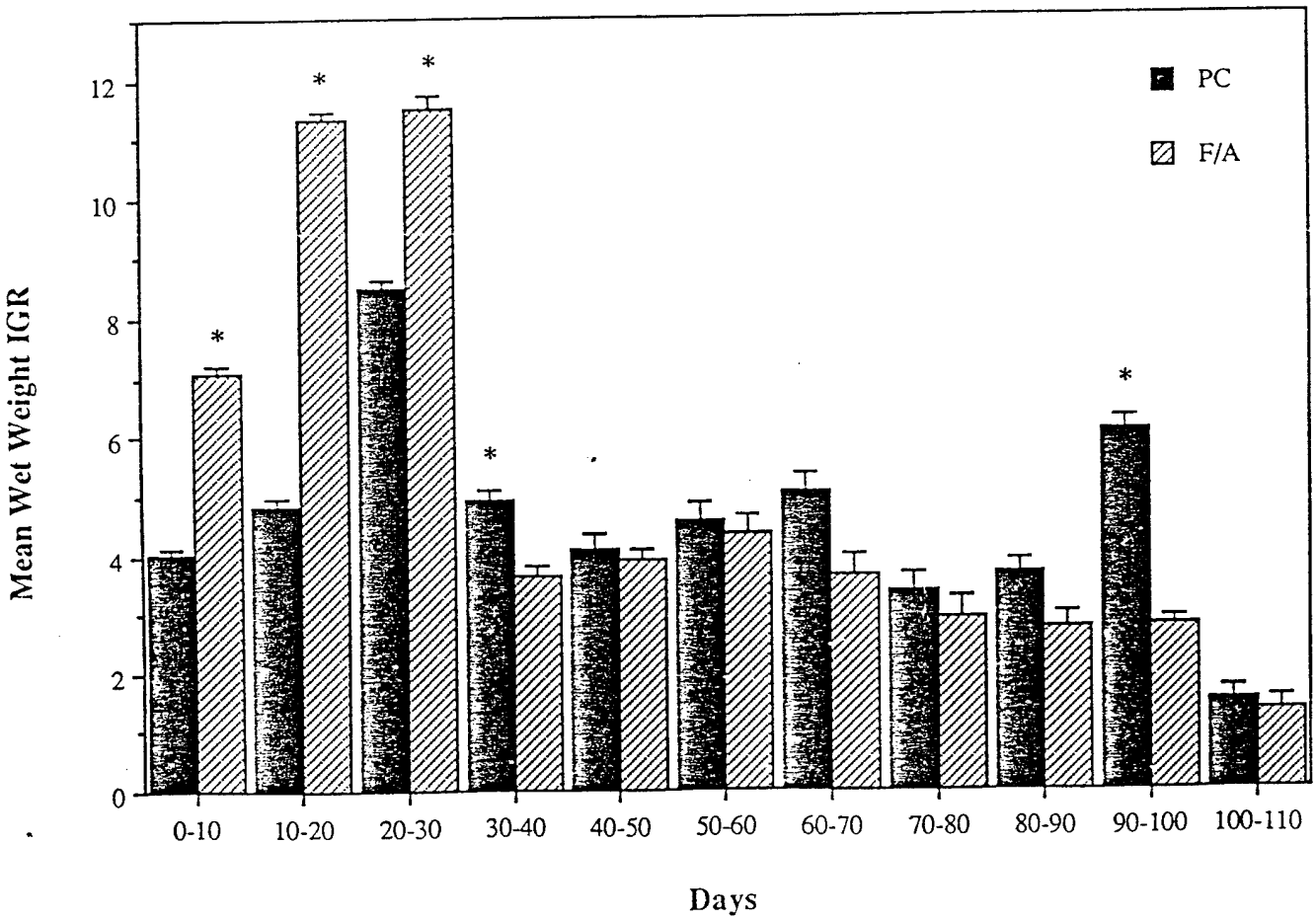
LEGENDS FOR FIGURES

Figure 1-2. Trial 2. Effect of diet on individual wet weight (Mean ± SE) of medaka. Standard errors that are not visible are contained within the symbols.


Flake & Artemia Diet
PC Diet
Individual Wet Wt. (g)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
70
80
90
100
110
Day

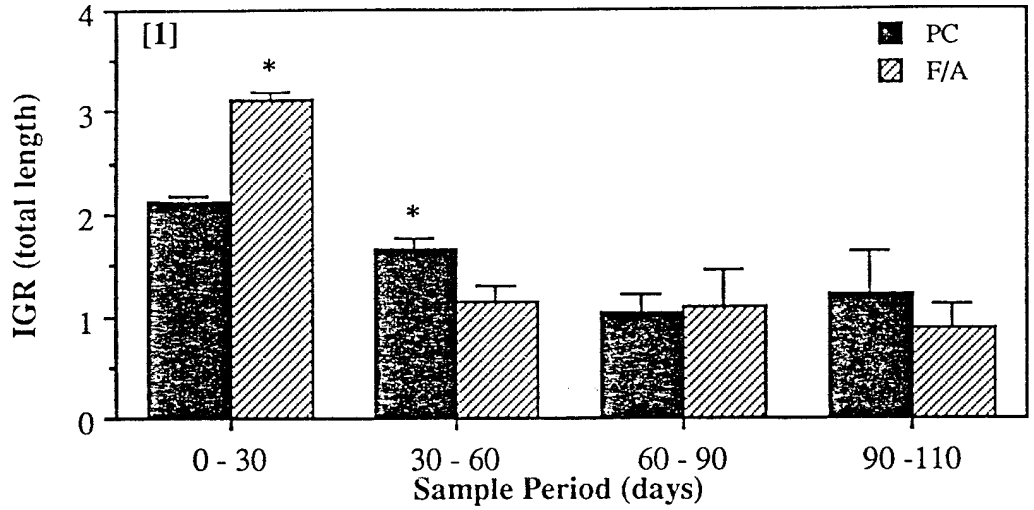
LEGENDS FOR FIGURES

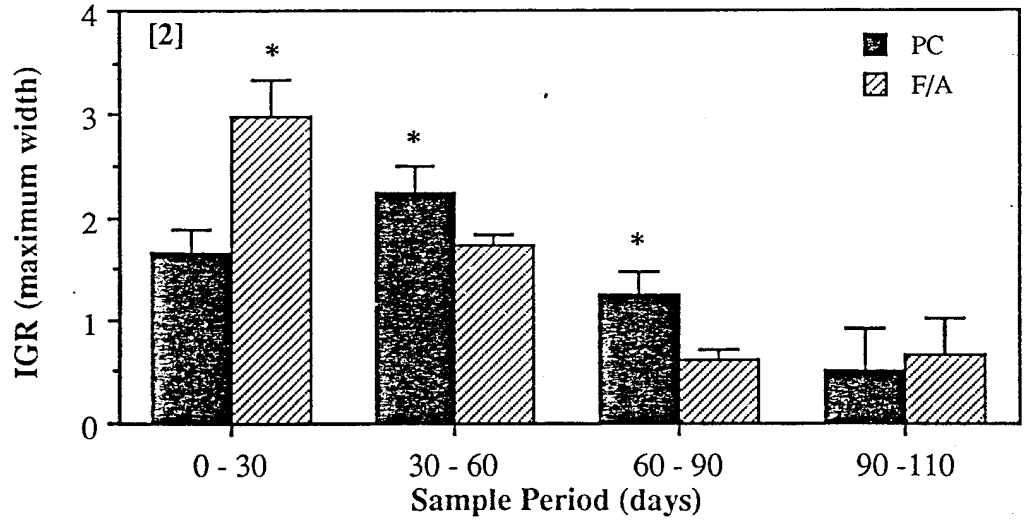
Figure 1-3. Trial 2. Average instantaneous growth rates (IGR; $\pm$ SE) using wet weight of medaka reared on Purified Casein (PC) and Flake/*Artemia* (F/A) diets. *Denotes IGRs within same sample period that are significantly different ($P \leq 0.05$).

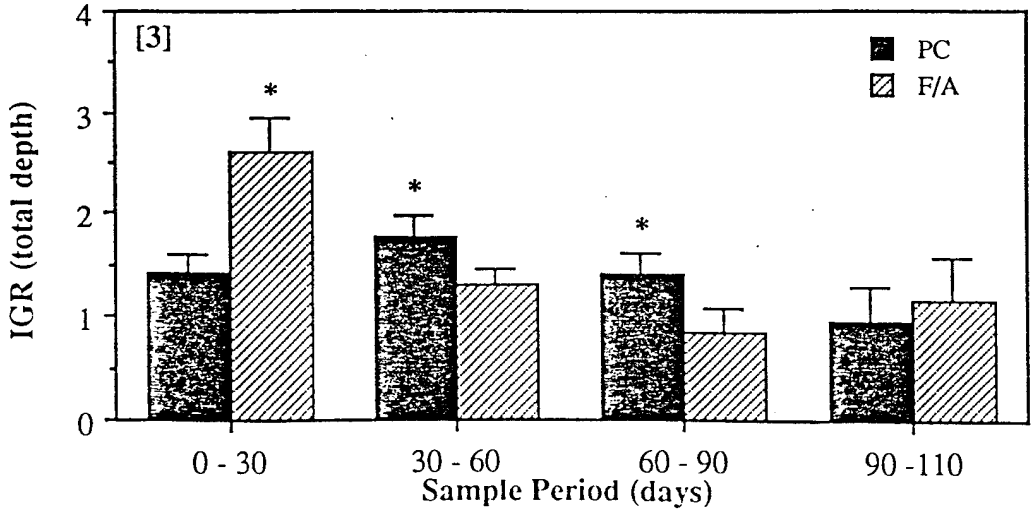

Mean Wet Weight IGR
0
2
4
6
8
10
12
PC
F/A
*
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100-110
Days

LEGENDS FOR FIGURES

Figure 1-4. Trial 2. Average instantaneous growth rates (IGR; $\pm$ SE) for total length [1], maximum width [2], and total depth [3] of medaka reared on Purified Casein (PC) and Flake/*Artemia* (F/A) diets. *Denotes IGRs within same sample period that are significantly different ($P \leq 0.05$).


[1]
PC
F/A
IGR (total length)
4
3
2
1
0
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)


[2]
PC
F/A
IGR (maximum width)
4
3
2
1
0
*
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)


[3]
PC
F/A
IGR (total depth)
4
3
2
1
0
*
*
*
0 - 30
30 - 60
60 - 90
90 -110
Sample Period (days)

LEGENDS FOR FIGURES

Figure 1-5. Normal medaka (Purified Casein, PC-diet) versus medaka with congenital "wavy-tail" and acquired Flake/*Artemia* (F/A) diet-related abnormalities. The fish with the "wavy-tail" was not part of the diet studies. Magnifications of gross photographs, radiographs, and mid-sagittal histologic sections vary slightly, but all three fish are 2 to 3 cm long.

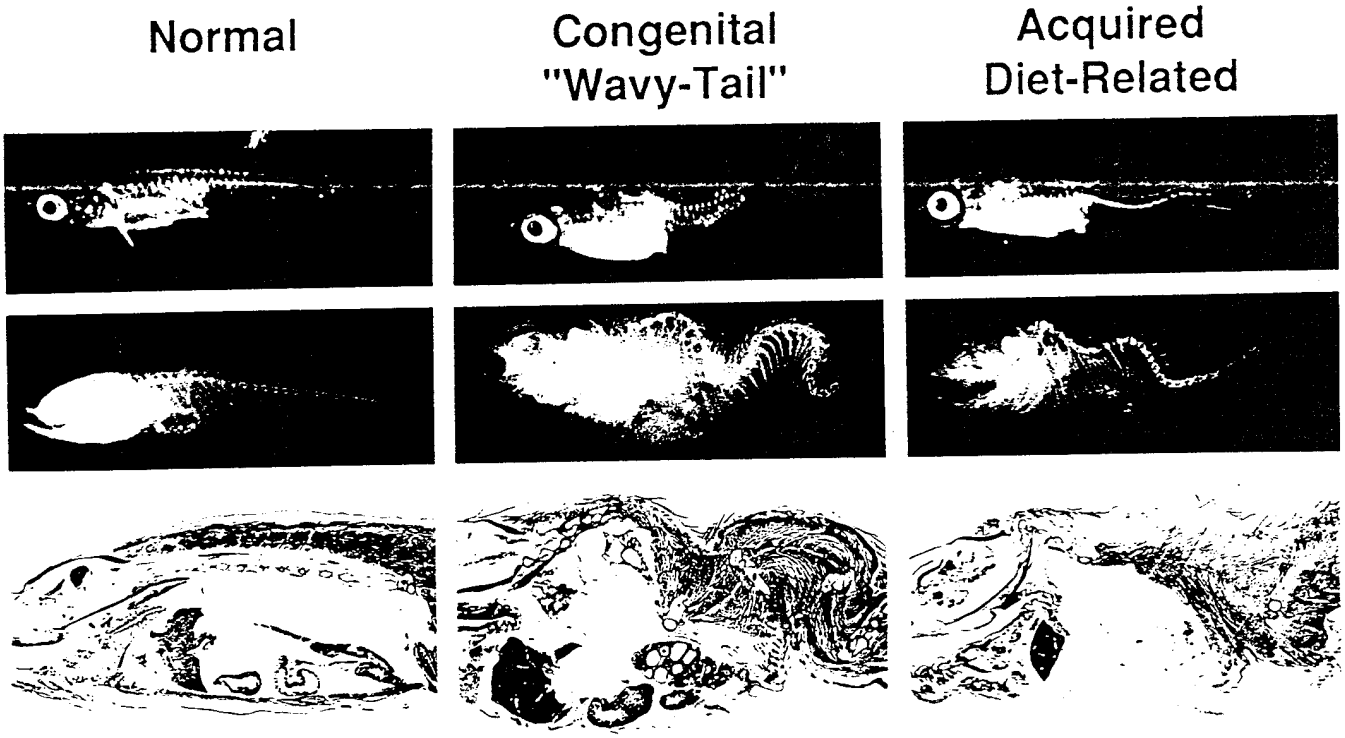

Normal
Congenital
"Wavy-Tail"
Acquired
Diet-Related

Table 1-1. Composition of Purified Casein (PC) diet for medaka

INGREDIENTS	PERCENT COMPOSITION
Vitamin-free casein	31.0
Wheat gluten	15.0
Dextrin	27.2
Refined soy lecithin	5.2
BML-2 vitamin mix (-C)[a]	4.0
Egg albumin	4.0
BTM mineral mix[b]	3.0
Non-nutritive bulk	3.53
Corn oil	2.0
Cod liver oil	5.0
Tert-butyl hydroquinone	0.002
Vitamin K	0.005
Vitamin A (500,000 IU/G)	0.0018
Vitamin D (400,000 IU/G)	0.00025
Vitamin E[c] (250 IU/G)	0.0075[d]
Vitamin C[e]	0.05

[a] Contains (mg/kg diet in alphacel): thiamin mononitrate (200), riboflavin (320), nicotinic acid (1,040), Ca-d-pantothenate (600), pyridoxine HCL (120), cobalamine (40), folic acid (200), biotin (40), myo-inositol (7,200), p-amino-benzoic acid (120), BHA (40).

[b] Contains (mg/kg diet): calcium carbonate (630), calcium phosphate (22050), citric acid (68.1), cupric citrate · 2½ H_2O (13.8), ferric citrate ·5H_2O (167.4), magnesium oxide (750), manganese citrate (250.5), potassium iodide (0.3), potassium phosphate dibasic (2430), potassium sulfate (2040), sodium chloride (918), sodium phosphate (64.2), zinc citrate · 2H_2O (39.9).

[c] As dl-α-tocopherol acetate.

[d] Vitamin E was increased to 0.03% in Trial 2 in accordance with NRC recommendations (48).

[e] In Trial 2, vitamin C was included in BML-2 Vitamin mix as ascorbic acid (500mg/kg)

Table 1-2. Trial 2. Proximate analysis of the diets, Flake (FL-diet), Purified Casein (PC-diet) and Artemia (A-diet).

	DIETS		
	FL-diet	PC-diet	A-diet[a]
Dry matter[b]	94.5	94.4	10.3
Crude Protein[c]	50.3	46.4	59.2
Ether Extract[c]	7.8 12.9	19.4	
Ash[c]	11.8	8.2	11.7

[a] Calculated from Watanabe *et al* (50)

[b] Dry matter as % of wet weight

[c] Chemical analysis as % of dry matter

Table 1-3. Trial 1. Average wet and dry weights (mean ± SE) of fish reared on Flake (FL), Purified Casein (PC) or live Artemia (A) diets at 24 weeks.

	FL-diet	PC-diet	A-diet
Wet Weight (mg)	73.58 ± 1.43*	107.33 ± 1.61	119.83 ± 0.99
Dry weight (mg)	16.98 ± 1.44*	26.81 ± 1.33	30.15 ± 0.77

*Significantly different ($p \leq 0.05$) from other values in the same row.

Table 1-4. Trial 1. Instantaneous growth rates [a] (mean ± SE; n=3) of medaka fed Flake (FL), Purified Casein (PC) or live Artemia (A) diets; weeks 0-7, 7-9, 9-12.

	WEEK		
	0-7	7-9	9-12
DIET:			
FL	4.88 ± 0.25*	3.45 ± 0.39	2.46 ± 0.40
PC	6.52 ± 0.78	4.01 ± 0.42	2.22 ± 0.60
A	7.03 ± 1.07	4.56 ± 0.40	1.73 ± 0.42

*Significantly different ($p \leq 0.05$) from other values in the same column.

[a] Instantaneous Growth Rate (IGR) = $\frac{LnW_f - LnW_i}{\text{\# days}} \times 100$

W_f = final wet weight, W_i = initial wet weight

Ln = natural log

Table 1-5. Trial 2. Morphometric analysis (total length, maximum width, and maximum depth (mean ± SE) at 0, 30, 60, 90, and 110 days in fish fed Purified Casein (PC) or Flake/*Artemia* (F/A) diets.

Sample	Total Length (mm)		Maximum Width (mm)		Maximum Depth (mm)	
day	F/A	PC	F/A	PC	F/A	PC
0	4.7 ± 0.02	4.7 ± 0.02	0.6 ± 0.01	0.6 ± 0.01	0.8 ± 0.009	0.8 ± 0.009
30	12.0 ± 0.02*	8.8 ± 0.02	1.5 ± 0.01*	1.0 ± 0.01	1.6 ± 0.03*	1.3 ± 0.007
60	16.4 ± 0.21*	13.8 ± 0.19	2.6 ± 0.02*	2.0 ± 0.01	3.0 ± 0.03*	2.2 ± 0.01
90	22.5 ± 0.13*	19.5 ± 0.12	3.0 ± 0.02	2.9 ± 0.02	3.8 ± 0.04*	3.3 ± 0.01
110	26.9 ± 0.09*	24.9 ± 0.21	3.3 ± 0.05	3.1 ± 0.02	4.8 ± 0.02*	4.1 ± 0.03

Values of five replicates (10 fish/replicate).

*Significantly different ($p \leq 0.05$) between the same set of morphometric parameters for the sample day.

Table 1-6. Enzyme activities (nmol/min/mg protein of triplicate assays) in pooled livers of medaka fed Flake/*Artemia* (F/A) and Purified Casein (PC) diets.

Age post-hatch (days)	ECOD		GST*	
	F/A	PC	F/A	PC
35	0.019 ± 0.002	0.022 ± 0.001	4.33 ± 0.110	4.64 ± 0.185
70	0.023 ± 0.001	0.019 ± 0.001	7.17 ± 0.358	7.51 ± 0.381
110	0.029 ± 0.002	0.027 ± 0.001	17.45 ± 0.860	28.80 ± 0.704**

* Activities of ethoxycoumarin O-diethylase (ECOD) and glutathione S-transferase (GST) are in the S9 pooled liver fraction from 20 medaka.

**Different from F/A pool by Spearman Rank Correlation test $P \leq 0.5$.

Chapter 2

Comparison of Hepatic Neoplasm Frequency in Medaka Fed a Purified Casein Based or Conventional Diet (Diethylnitrosamine)

Background

Progress to date has shown that the purified casein diet (PC) is capable of supporting medaka from hatch through egg production and that the progeny of adults fed only this diet are healthy fish. This accomplishment has largely met the initial objective of this contract. Now we moved to the next major objective which was to compare tumor incidence (frequency) between medaka fed a conventional ration (Tetramin flakes, Tetrawerke, Germany) plus two days supplementation each week with brine shrimp (*Artemia sp*) nauplii and those fed the PC-diet.

At present, medaka under carcinogen bioassay at various laboratories receive a variety of different diets. We have consulted with Dr. Keith Cooper at Rutgers University, their laboratory typically feeds Tetramin flakes alone or with brine shrimp supplementation. The United States Environmental Protection Agency, Environmental Research Lab at Duluth, Minnesota feeds medaka under test brine shrimp nauplii only (Personal Communication from Dr. Rodney Johnson). The Gulf Coast Marine Research Laboratory feeds day-old hatchlings an infusoria of live organisms suitably sized for uptake by young medaka. This initial feeding is continued for a period of approximately two weeks after which the fish receive brine shrimp nauplii and are eventually moved to a commercially available ration supplemented with ocean plankton (Personal Communication from Dr. W. Walker).

The formulation of commercial diets are typically proprietary and constituents may vary

from batch to batch. Even the components may be designed more for production than for rigid tests needed to compare nutritional modulation of carcinogenesis or to provide the consistency between tests which are needed for statistical evaluations. When natural (live) foods are used, the possibility of addition of adventitious xenobiotics arises.

We hypothesize that xenobiotics might alter the post-initiation phases of carcinogenesis (i.e., promotion and progression). It is entirely possible that the time to tumor, as well as the tumor frequency, will vary as a function of the diet. We therefore ultimately plan to use two different carcinogens under different times and routes of exposure and to determine the neoplastic potential of each. To meet this goal, very young medaka (21 days post-hatch) are briefly (48 hours) exposed to an aqueous solution of 350 ppm of diethylnitrosamine (DEN). Serial bioassay at monthly intervals was then used to determine the frequency of tumor formation. In this chapter, we report on our progress through seven months comparing tumor frequency in medaka fed the PC-diet or the flake plus *Artemia* (F/A) diet from day one of hatch and exposed to a brief pulse of DEN at 21 days.

Materials and Methods

Fish

Broodstock of golden variety medaka were maintained at 25°C under a 16 L:8D photoperiod. Eggs from several females were pooled and incubated in modified (no methylene blue), aerated, embryo rearing media (Kirchen and West, 1976; Rugh, 1962) at 25±1°C. Medium was replaced daily at which time any dead embryos were removed. Hatch took place at 9 to 10 days after fertilization. All fish were reared in a recirculation aquarium system using reconstituted water prepared following EPA guidelines (Horning and Weber, 1985) for

moderately hard water. Reconstituted water was prepared in batches of 500 gallons using reverse osmosis produced water as a feed. Water temperature was maintained at 25±1°C. Ammonia and nitrite levels were monitored weekly and maintained at less than 0.1 ppm. Nitrates, pH, conductivity and hardness were monitored weekly. Dissolved O_2 was maintained at or near saturation.

Newly hatched larvae with normal swimbladder inflation were randomly sorted and equally distributed among aquaria. To reduce bias in selection of fish, a 10-inch wide net was used to trap and concentrate approximately 100 fish. From this pool, and while the fish remained immersed, individuals were collected with a glass beaker and randomly assigned, in series, to the aquaria. The process was repeated serially until the desired number of fish was obtained for each aquarium. This process permitted selection of healthy individuals and overcame bias due to capture/evasion effect. An assistant was given 4 identically sized, sealed envelopes of uniform appearance and asked to place one on each aquarium. A card, inside each envelope, designated the diet for fish within that aquarium. Fish were fed to slight excess twice daily and tanks were siphoned to remove excess food and feces.

At 21 days of age, normal and healthy juveniles, of uniform size, (500 fish per treatment) were selected as described above. Also, 100 fish were selected from each diet for the control group at this time. Abnormal or weak fish were discarded. Selected fish, in labeled containers corresponding to diet group, were then transported to our exposure facility.

Medaka from each diet group scheduled for exposure to diethylnitrosamine (DEN) were placed in 10-L glass aquaria (density = 50 fish/L) and exposed for 48 hr to an aqueous bath of 350 ppm DEN. Actual concentrations of DEN in exposure water were determined at 0, 12 and

24 hours. After this, total replacement with fresh reconstituted water plus DEN (350 ppm) was done. Similarly, assay for DEN concentration was done immediately after mixing in the aquaria and at 36 and 48 hours. Except for DEN, control fish were subjected to the same conditions as the exposed fish and placed in a glass aquarium with 2-L of reconstituted water (density = 50 fish/L). After exposure, fish were transferred to clean reconstituted water and then feeding with their respective previous diets was initiated. A brief duration was selected in order to insure homogeneity of DEN concentration within aquaria. This regime has been used in our laboratory to produce hepatocellular carcinoma in medaka. The brief initiation followed by somatic and liver growth, considered as promotional factors, enables a more thorough comparison of the effect of diet on promotion and progression of hepatic neoplasms. Aquarium water was tested for residual DEN, when none was detected fish were transferred to the recirculating system in our rearing facility. Exposed fish were then placed in two 20-gallon aquaria, 250 fish per aquarium. Control fish were placed in one 10-gallon aquarium. Fish were allowed to "grow out" under the same conditions and previous diet regimes. Selection of rack and placement within racks for the 4 aquaria containing exposed fish was done in a randomized fashion using sealed envelopes as described above.

Quantification of DEN was by spectrophotometric analysis at a wavelength of 230 nm (IARC, 1981), of water taken directly from the aquaria. Standards were prepared with reconstituted water and ranged from 0.1 to 10 ppm. By direct comparison with gas chromatography, our previous work had shown that the spectrophotometric method has proven to be reliable and faster.

Particle sizes of the formulated rations were increased as the fish grew. From hatch to

4 weeks, fish were fed formulated rations ranging from 100 to 250 µm particle diameter. After 4 weeks, the fish were fed particle sizes ranging from 250 to 850 µm diameter. Newly hatched brine shrimp were separated from unhatched and empty cysts and rinsed with distilled water prior to being fed to fish (*Artemia* F/A group only). *Artemia* F/A group was fed flaked food five days per week and brine shrimp nauplii two days (Tuesday and Friday) each week. Fish in the other two aquaria were fed only the purified casein (PC) diet (DeKoven, et al., 1992).

Each month, fish were randomly sampled (10 per replicate, total of 20 per treatment-exposed fish; 4 per treatment-control group) for wet weight determinations and general histology. Collected fish were anesthetized in MS-222, placed in Bouin's fixative for 48 h, dehydrated in graded alcohol solutions and processed for paraffin embedment. Sections of paraffin embedded material were cut at 6 µm and stained with hematoxylin and eosin (H&E). Histologic analysis was performed to enumerate tinctorially altered foci, adenoma, cholangioma, hepatocellular carcinoma, cholangio-cellular carcinoma and mixed cell (both hepatocytes and biliary epithelial cells) tumors. The above histologic methods closely followed procedures which have been used in this lab in previous studies (Hinton, et al., 1987; Laurén, et al., 1990).

Results and Discussion

Growth: Both control groups showed greater growth than their respective exposed groups (Tables 7a and b and Figure 6). Although the changes in weight have been monitored through 180 days, fish exposed for 48 hrs to DEN continue to show less growth than controls and the clustering of the two exposed diet treatments (Figure 6) suggests that it is DEN and not the particular diet which is responsible for the altered growth pattern. By comparison, PC-diet fed controls showed a trend toward greater growth during the 90-150 day interval than did the control

medaka fed the flake *Artemia* regimen (Figure 6).

Mortality: Two DEN-exposed fish, one from each diet group, died during the 48 hr exposure. No control deaths were recorded during the 48 hr period (Table 8a). Subsequently, 95 fish (19% mortality) died in the PC-fed and DEN-exposed group. Apparent lower mortality (60 fish, 12%) was seen in the flake *Artemia*-fed DEN-exposed group (Table 8b). The histogram (Figure 7) graphically represents actual numbers of dead fish in individual "grow out" aquaria. We observed no "tank-specific" effect. Mortality data suggest that both groups are showing DEN-induced toxicity with the PC-diet group showing possible greater effect.

Histopathology: Control hepatic alterations were minimal throughout the study. After the initial 48 hrs of test, controls corresponding to this time interval showed no lesions. After 1 month, a single PC-diet-fed control fish showed focal perivascular necrosis and spongiotic change. No lesions were seen in the F/A-fed control fish at one month. At two months, a single F/A-fed fish showed a single vacuolated focus similar to that shown in Figures 8 and 9. Similarly, two PC-fed control fish showed spongiotic change. At month three, PC-fed controls showed focal spongiotic change (3 of 4 examined). F/A-fed controls showed no lesions at month three. In month four, one F/A-fed control showed focal spongiosis hepatis. Corresponding PC-fed controls were lesion free. All control fish from months five, six and seven were lesion free. Spongiosis hepatis has been reported in carcinogen-exposed rats (Bannasch, et al., 1981) and in medaka (Hinton, et al., 1984a) and *Cyprindon variegatus* (Couch, 1991). The lesion has also been reported at low incidence in multi-year control fish. In the present study, the lesion may be involved in growth-related hepatic remodeling (Wyllie, et al., 1980). Except for the single vacuolated focus (in flake *Artemia*-fed control at month two), control livers have been free

of foci and neoplasms.

Classification Nomenclature - Hepatocellular Neoplasms and Early Lesions

There are no published guidelines for nomenclature of fish, specifically medaka, hepatic neoplasms and associated lesions. Rather, a collection of various terms and lesion descriptions exists usually as a small section in each of the original papers. Rodent bioassays have adopted uniform criteria by which alterations are classified (Boorman, et al., 1990; Maronpot, et al., 1986). The adoption of such criteria, while facilitating uniformity of bioassay results, must reflect the breadth and nature of histopathologic alterations. I have contributed a chapter on normal morphology and early fish hepatic alterations encountered after laboratory exposure to carcinogens (Hinton, In Press). This laboratory has contributed much to our understanding of histopathologic biomarker lesions (Hinton, 1993; Hinton, et al., 1992b; Hinton, et al., 1984a; Hinton, et al., 1987; Hinton and Laurén, 1990a; Hinton and Laurén, 1990b; Hinton, et al., 1984b). In response to needs expressed at the 1991 Histopathologic Workshop on liver lesions of fishes exposed to carcinogens, United States Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida, and at the recent National Toxicology Program meeting on Fish Carcinogenic Models, we have devoted efforts under this contract to describe and illustrate medaka liver histopathology associated with DEN-induced carcinogens. Table 9 presents key features of the medaka classification system for foci and neoplasms, the principal lesions of this study. Only those lesions visible with H&E staining were enumerated.

Regardless of the diet, DEN-exposed fish developed foci and neoplasms further supporting our earlier studies with this compound in medaka (Hinton, 1989a; Hinton, et al., 1988b; Hinton, et al., 1985; Hinton, et al., 1988a; Hinton, et al., 1992a). Alterations encountered after medaka

are exposed to DEN include an early hepatotoxicity primarily directed at hepatocytes. Lesions of an early toxic nature characterized all DEN-exposed fish of this study. These have been described in detail at both the light and electron microscopic levels (Braunbeck, et al., 1992; Laurén, et al., 1990).

The foci and neoplasms of this study followed the early toxic response. After one month, 14 foci were seen in PC-fed DEN-exposed medaka (1 basophilic, 4 clear cell and 9 vacuolated). Corresponding F/A-fed fish showed a total of 9 foci (5 basophilic and 4 vacuolated). After two months, PC-diet fish showed 18 foci (2 eosinophilic, 2 clear cell and 14 vacuolated). In F/A-fed fish, 20 foci were seen at this time (3 eosinophilic, 2 clear cell, 15 vacuolated). By the end of 3 months, PC-fed fish showed 11 foci (2 basophilic, 4 clear cell, 5 vacuolated). At this time, F/A-fed fish showed 35 foci (1 basophilic, 2 clear cell and 32 vacuolated). Month four fish showed 10 foci (PC-diet; 5 basophilic, 3 eosinophilic, 9 clear cell, 2 vacuolated). F/A-fed fish at this month showed 21 foci (1 basophilic, 4 eosinophilic, 16 vacuolated). At month five, PC-fed fish showed a total of six foci (1 eosinophilic, 3 clear cell and 2 vacuolated). Month five F/A-fed fish showed 16 foci (6 eosinophilic, 2 clear cell and 8 vacuolated). Month six medaka fed PC-diet showed 40 foci (3 basophilic, 5 eosinophilic, 1 clear cell and 21 vacuolated). Corresponding F/A-fed fish showed a total of 9 foci (2 eosinophilic and 7 vacuolated). In month 7 there were 37 foci in PC-fed medaka (8 basophilic, 7 eosinophilic, 2 clear cell and 20 vacuolated). F/A-fed fish at month 7 showed 25 foci (4 basophilic, 4 eosinophilic, 14 clear cell, 3 vacuolated). Statistical evaluation of the data will follow the final sampling for histopathologic analysis and does not fall within the first half of this project.

The foci enumeration should be regarded as semiquantitative since no morphometric

procedures (Weibel, 1980) were followed. These are to be done in the remaining two years. However, foci preceded neoplasms suggesting similarity in progression between rodent (Farber, 1976; Pitot, 1983) and medaka (Hinton, et al., 1988b) hepatocarcinogenesis. Of the medaka foci, basophilic and eosinophilic are generally regarded as the most relevant to tumorigenesis. A basophilic focus found in a liver section from a PC fed, DEN-treated, female medaka at six months after onset of exposure is shown in Figure 8. The major difference of cells within the focus versus their counterparts in the "noninvolved liver" are related to the staining within the cytoplasm. The architectural arrangement of surrounding liver and focus is nearly identical with the latter perhaps showing slight enhancement of the tubular arrangement (Fig. 8). Figure 9 illustrates features of an eosinophilic focus. This particular focus was seen in the liver section of a female medaka fed the F/A diet for 6 months. Component hepatocytes of eosinophilic foci differ appreciably in size (Fig. 9). Features of a clear cell focus from the liver of a female medaka at 5 months after the onset of exposure are seen in Figures 10 and 11. This fish was fed the PC-diet. Cells of clear cell foci show the least staining over cytoplasm in H&E stains. They are followed by normal, glycogen enriched cells and then by the cells of eosinophilic and basophilic foci. Abrupt margins where cells with focal staining characteristics abut on cells with normal features characterize most foci (Figs. 8,9,10,11). A vacuolated focus is shown in figure 12. This lesion was seen in the liver of a PC fed, female fish at 7 months after the onset of exposure. By contrast with figures 10 and 11, the large vacuoles of smooth outer contour and eccentric nuclei differ from the appearance of clear cells. Vacuolated cells are regarded as fat filled hepatocytes. No diet specific differences among a single category of focus were encountered. The fate of the numerous vacuolated foci needs attention. Their occurrence in both

control and treated livers is akin to certain "spontaneous" foci in rodent liver (Pitot, et al., 1989). Our future work will be to quantify initial foci number and then to follow growth of these lesions. Once data is derived on number and growth, we can proceed with testing to determine initiation and promotion indices (Pitot, Campbell, et al. 1989) for individual compounds and complex mixtures.

Resultant neoplasms, by dietary group, are presented in sequential fashion (Table 10). Both rations proved sufficient to support tumor formation in DEN-exposed medaka. However, it appears that greater tumor yields may result from use of the F/A ration. Whether this is related to progressional and/or promotional factors not present in the PC-diet remains to be tested. The time to first tumor appears to be identical whether fish were fed PC or F/A diets (Table 10). However, from 3 months on, a total of 14 and a total of 19 tumors resulted in PC and F/A groups, respectively (Table 10). It is too early to tell whether tumor promotional and/or progressional agents are present in the F/A ration. Complete statistical analysis will follow at the termination of the study.

Histopathologic analysis of a male medaka exposed to DEN for 48 hrs (350 ppm) and fed the F/A diet revealed the presence of a cholangioma at 6 months after onset of exposure (Fig. 13). Architecture of the biliary passageways retains a differentiated state. However, the profiles of ducts are very numerous in this lesion. In addition, the fairly typical nuclei are beginning to "pile up" in multiple rows. There is no evidence that the neoplasm has invaded the adjacent parenchyma.

A cholangiocarcinoma is shown (Fig. 14). This neoplasm was found in the liver of a male medaka fed the F/A diet. The neoplasm was encountered at 4 months after the onset of

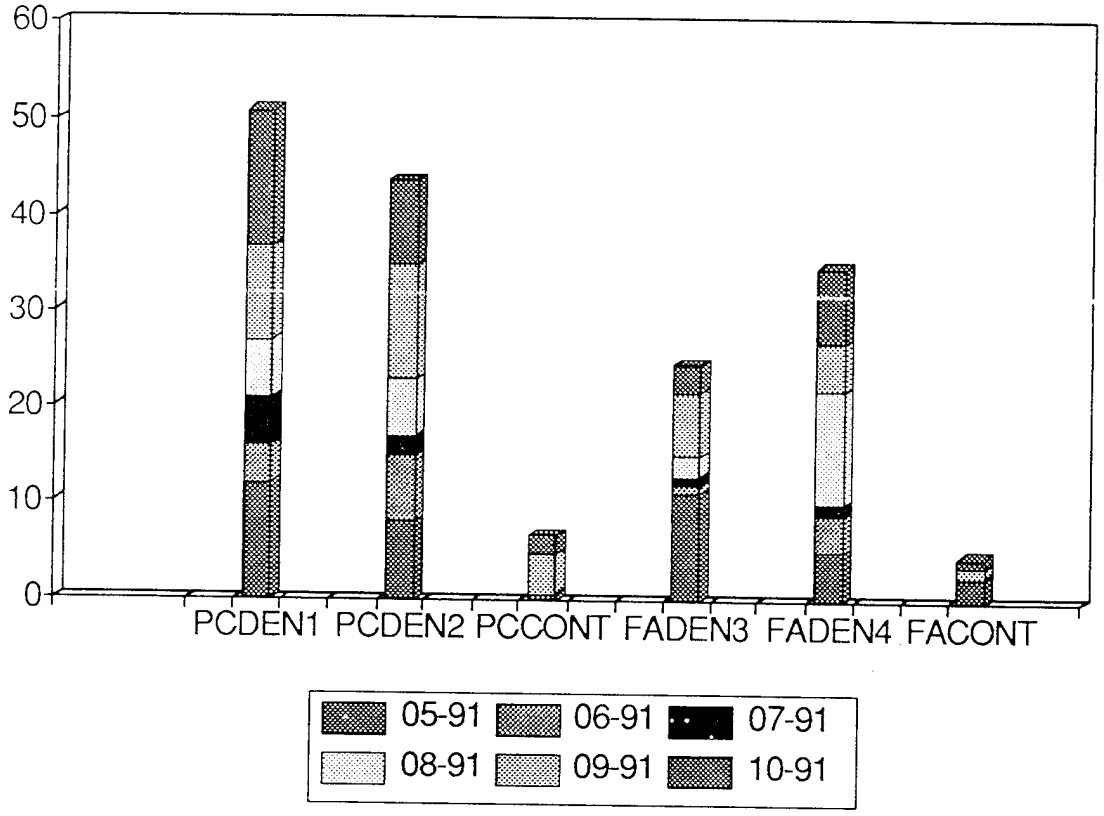
exposure to DEN (48 hr bath at 350 ppm concentration). By contrast with figure 13, the cholangiocarcinoma has invaded the adjacent parenchyma. Both ductular and trabecular patterns are indicated. Numerous mitotic figures are present (Fig. 14).

A mixed hepato- and cholangiocellular tumor is shown in Figure 15. This neoplasm was detected in the liver of a female medaka at three months after the onset of exposure to DEN. This particular neoplasm was predominantly cholangiocellular with hepatic parenchymal tubules between ductular elements. Elements of a solid hepatocellular carcinoma are shown in figures 16 and 17. In the low magnification view (Fig. 16), the solid features and basophilic staining contrast with the remainder of the liver. Under higher magnification, nuclear pleomorphism and transformation of tubules into broad sheets of tumor cells are apparent (Fig. 17). This tumor was detected at seven months after onset of exposure and was in the liver of a male medaka fed the F/A diet.

At seven months after onset of exposure, a female medaka fed the F/A diet was shown to have developed a large hepatocellular carcinoma (Figs. 18 and 19). This lesion occupied the majority of the liver section and had a large necrotic component in its center (Figs. 18 and 19). Tumor also contained foci of spongiosis hepatis (Fig. 19).

Data through this midterm point indicate that the PC-diet will prove adequate as a single ration for medaka. The consistency of the open-formula, purified diet now makes it possible for us to pursue modulatory effects of diet and environment on fish liver carcinogenesis.

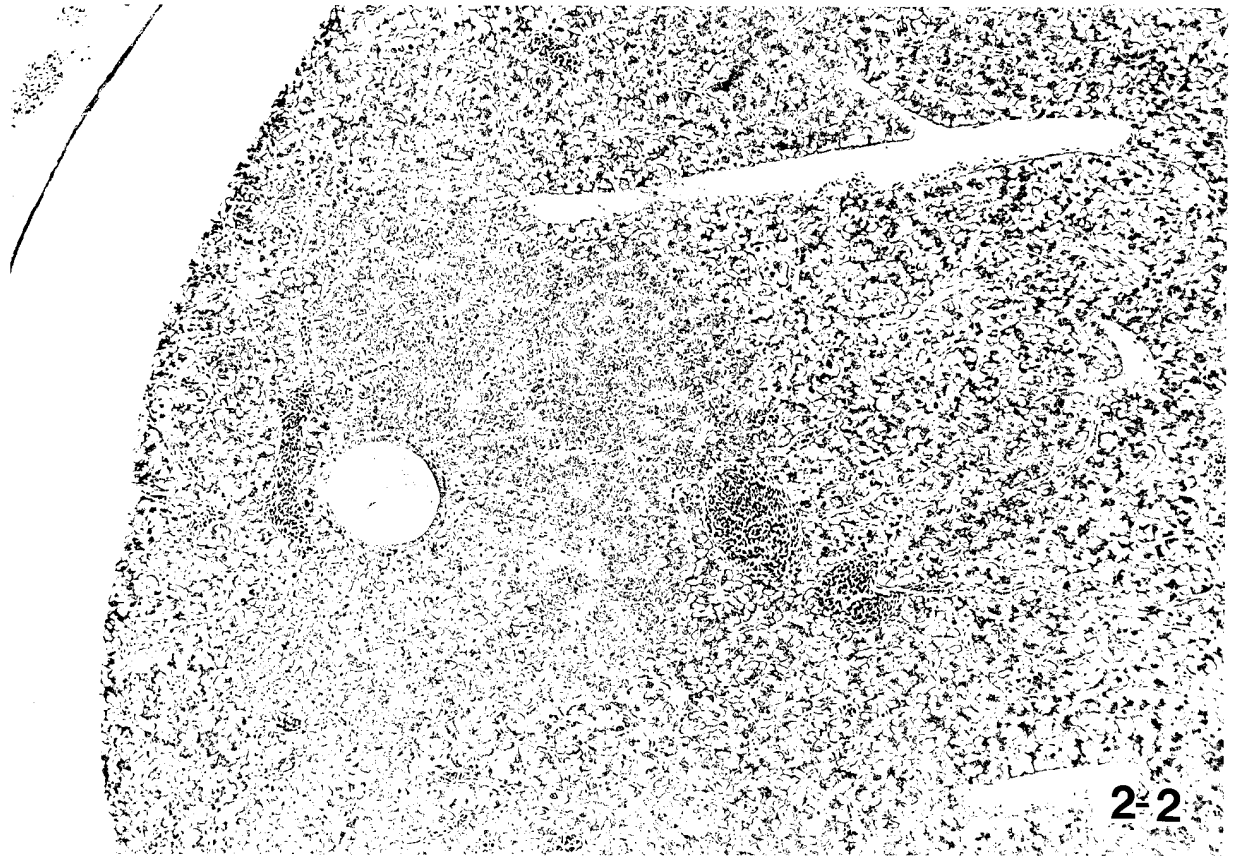
Figure 2-1. Mortality in Individual Aquaria



LEGENDS FOR FIGURES

Figure 2-2. This basophilic focus, of approximate lobular dimension, (325 μm diameter) contains dark-staining basophilic hepatocytes. Note similarity of cellular dimensions in focus and in surrounding hepatocytes. Tubular architectural pattern of hepatic parenchyma is enhanced in this focus. Female medaka fed the F/A diet and exposed for 48 hrs to 350 ppm DEN. Fish was fixed at 6 months after onset of exposure. Hematoxylin and eosin stain X 112.

Figure 2-3. Eosinophilic focus deep within liver section shows some enlarged hepatocytes and others of near normal dimensions. Component cells of focus illustrate two variants, the granular eosinophilic cells (large arrows) and the eosinophilic cell with glycogen remnants (small arrows). The majority of the cells are hypertrophic and multiple nucleated forms are seen. Female medaka fed the PC-diet before and after a 48 hr bath exposure to 350 ppm DEN. Lesion was detected at 6 months after onset of exposure. Hematoxylin and eosin stain X 225.



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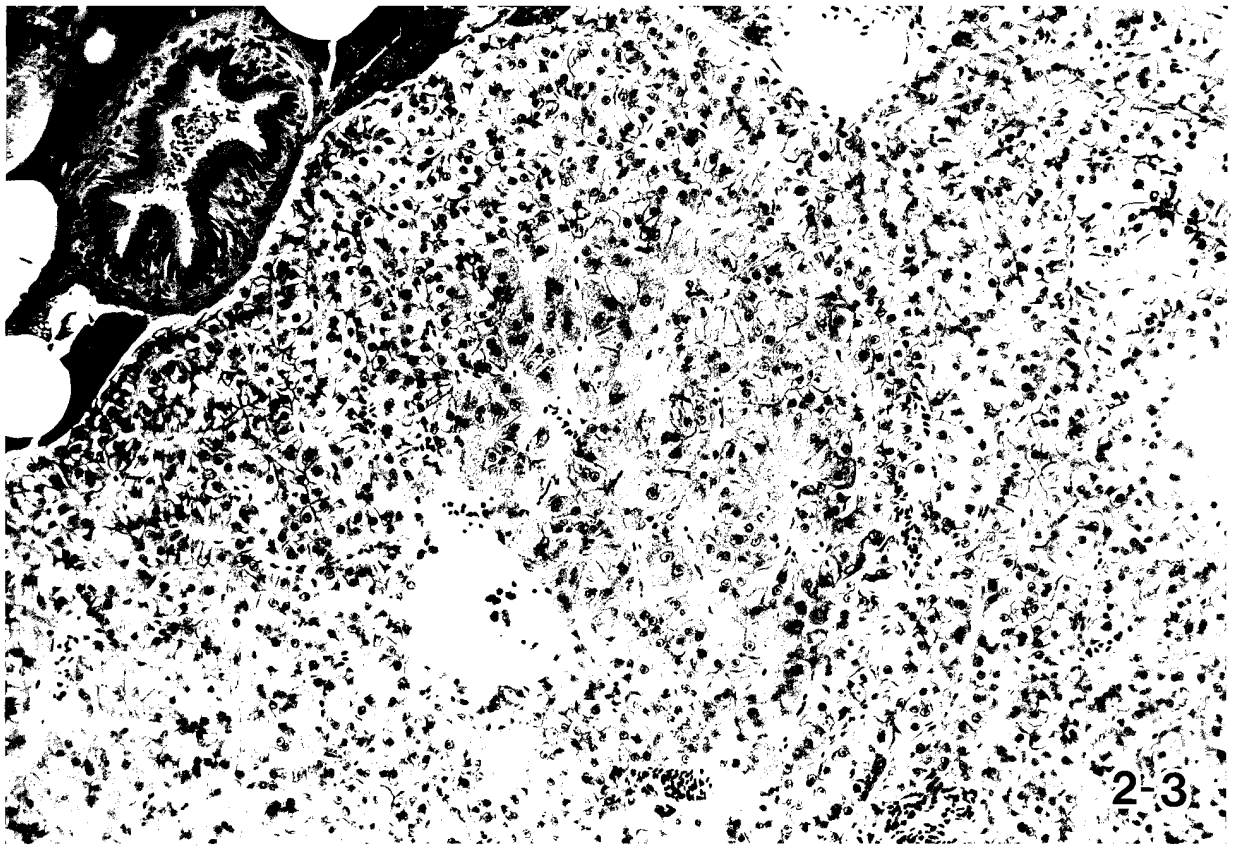
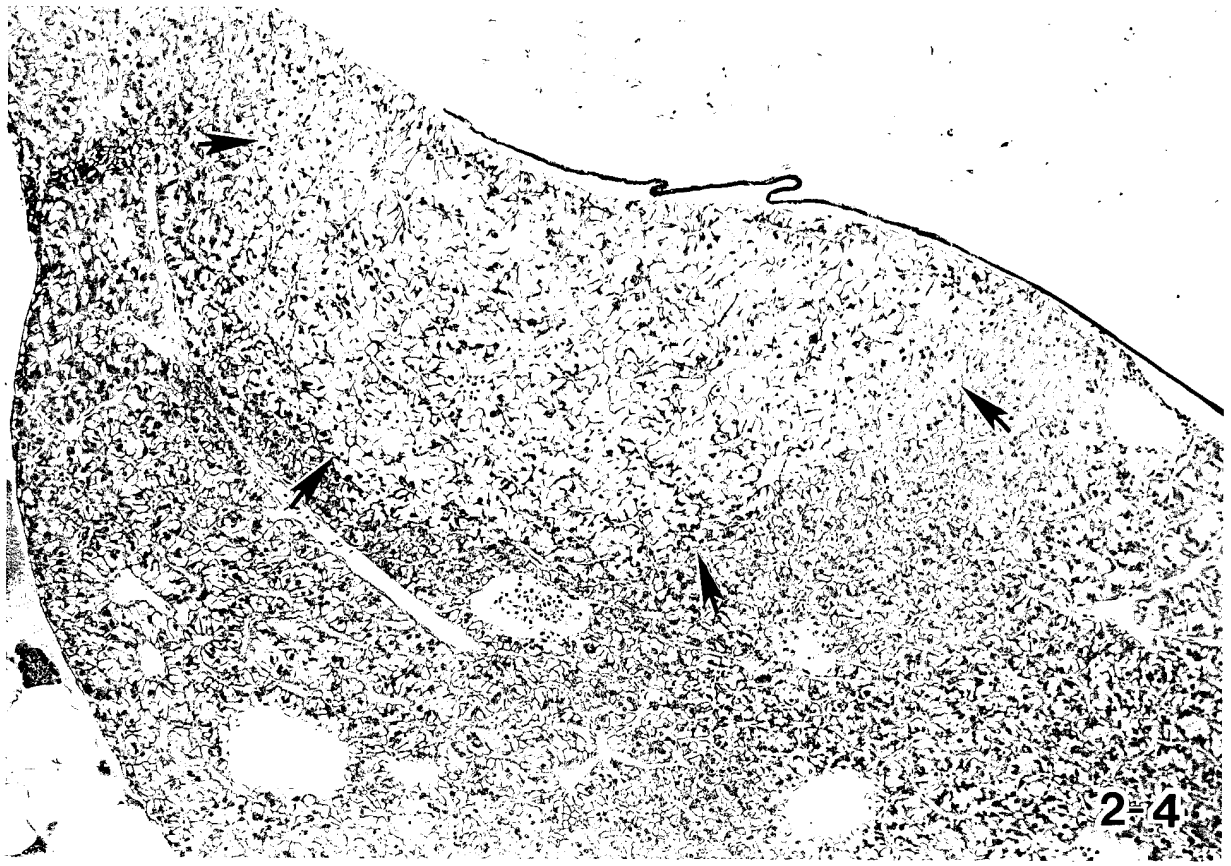


2-3

LEGENDS FOR FIGURES

Figure 2-4. Clear cell focus (arrows) at margin of liver section. Cells of foci appear larger than the surrounding cells. Histochemistry in prior companion studies shows glycogen in "clear" areas. Margin is indicated by arrows. This lesion appeared in the liver of a female medaka fed the PC-diet. Fish was exposed for 48 hrs to 350 ppm DEN and lesion was detected at 5 months after initiation of exposure. Hematoxylin and eosin stain X 112.

Figure 2-5. Higher magnification view of the focus illustrated in fig. 10. Cells of focus are larger and stain less than adjacent cells at bottom of field (arrows). Within individual clear cells, observe absence of smooth, rounded margins characteristic of fat vacuoles. A light gray material (C) surrounds clear areas within clear cells. This represents remaining elements in cytoplasm which take up stain. See legend for figure 2-4 for details of diet and exposure. Hematoxylin and eosin stain X 225.



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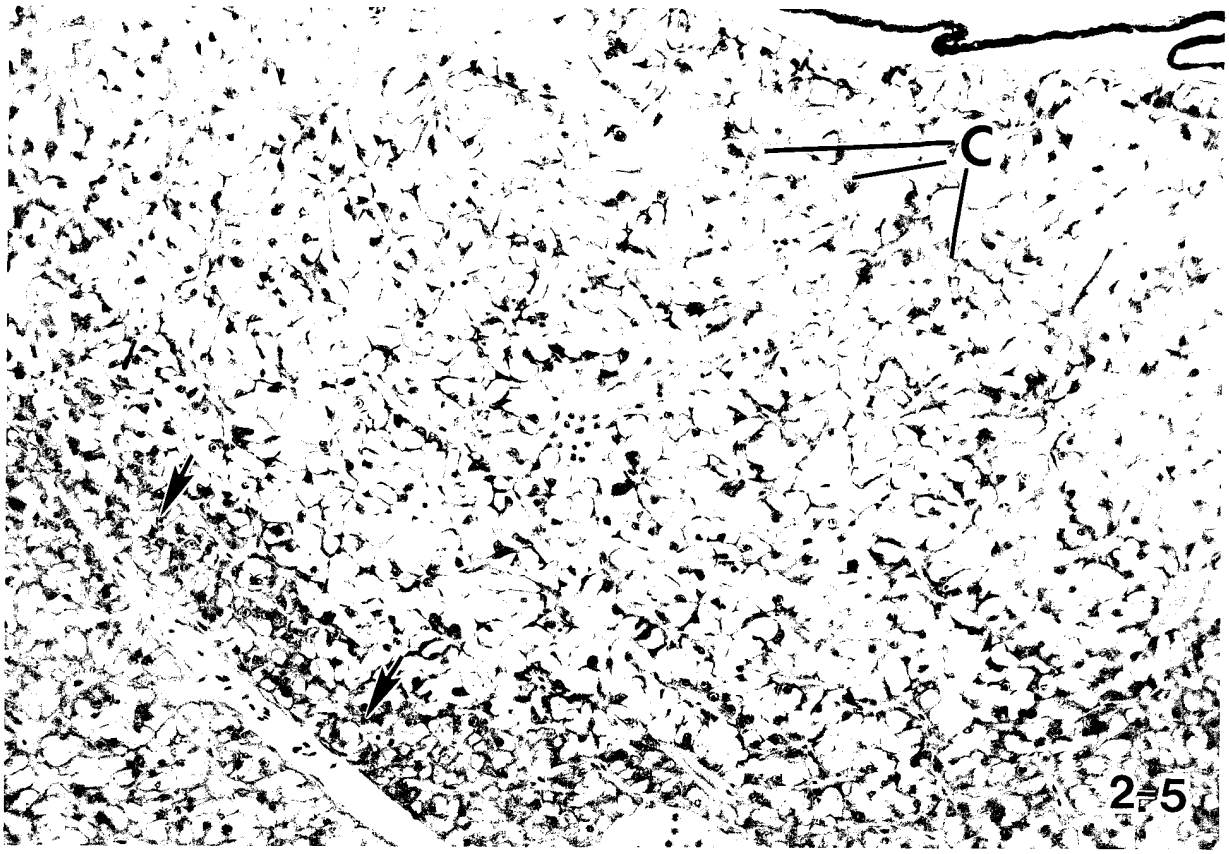
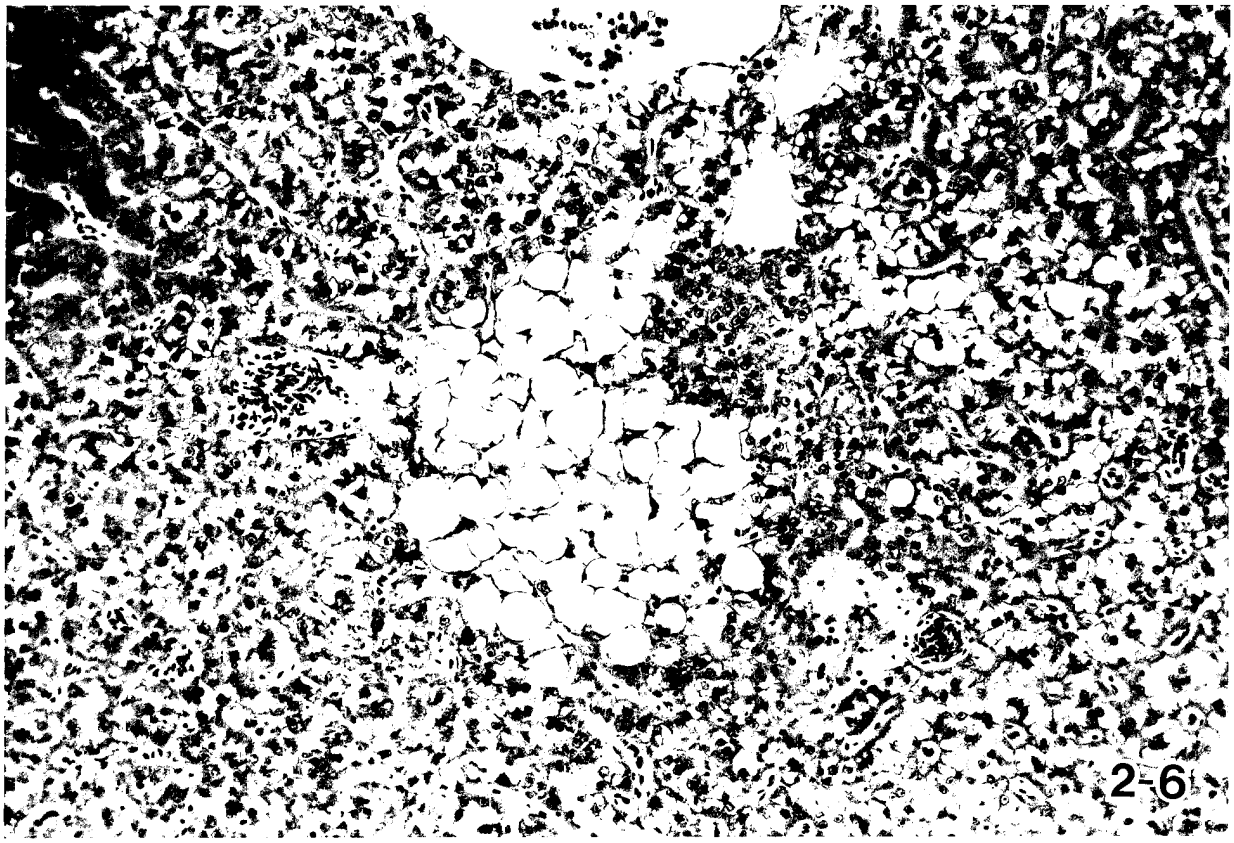


2-5

LEGENDS FOR FIGURES

Figure 2-6. Vacuolated focus reveals large vacuolar profiles with some confluence. Compare with figs. 2-4 and 2-5 to observe differences in vacuolated and clear cell foci. Nuclei of cells within this focus show peripheral displacement. This type focus is occasionally encountered in control fish but more often in DEN-treated animals. This lesion was detected at 7 months after onset of exposure (48 hr bath 350 ppm DEN). Fish was a female and was fed the PC-diet. Hematoxylin and eosin stain X 225.

Figure 2-7. Cholangioma involving intrahepatic bile ducts. Columnar to cuboidal epithelial cells continue to form mural elements of biliary passageways, however, "piling up" of nuclei is seen. Early stages of nuclear atypia are indicated by large elongated nuclei (arrows). All epithelial cells continue to appear surrounded by their basal laminae and no invasion of parenchyma is apparent. Male medaka fed the F/A diet and sampled at 6 months after onset of a 48 hr bath exposure to 350 ppm DEN. Hematoxylin and eosin stain X 225.



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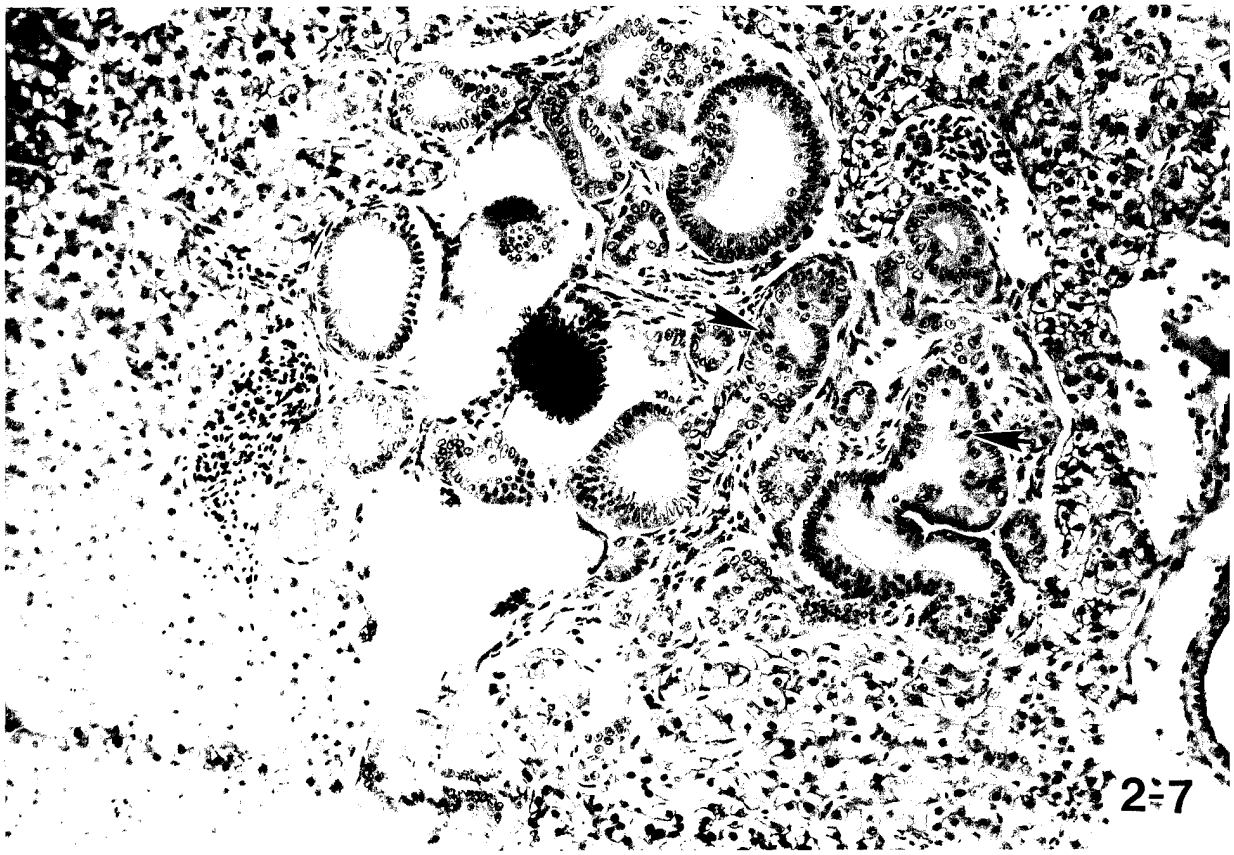
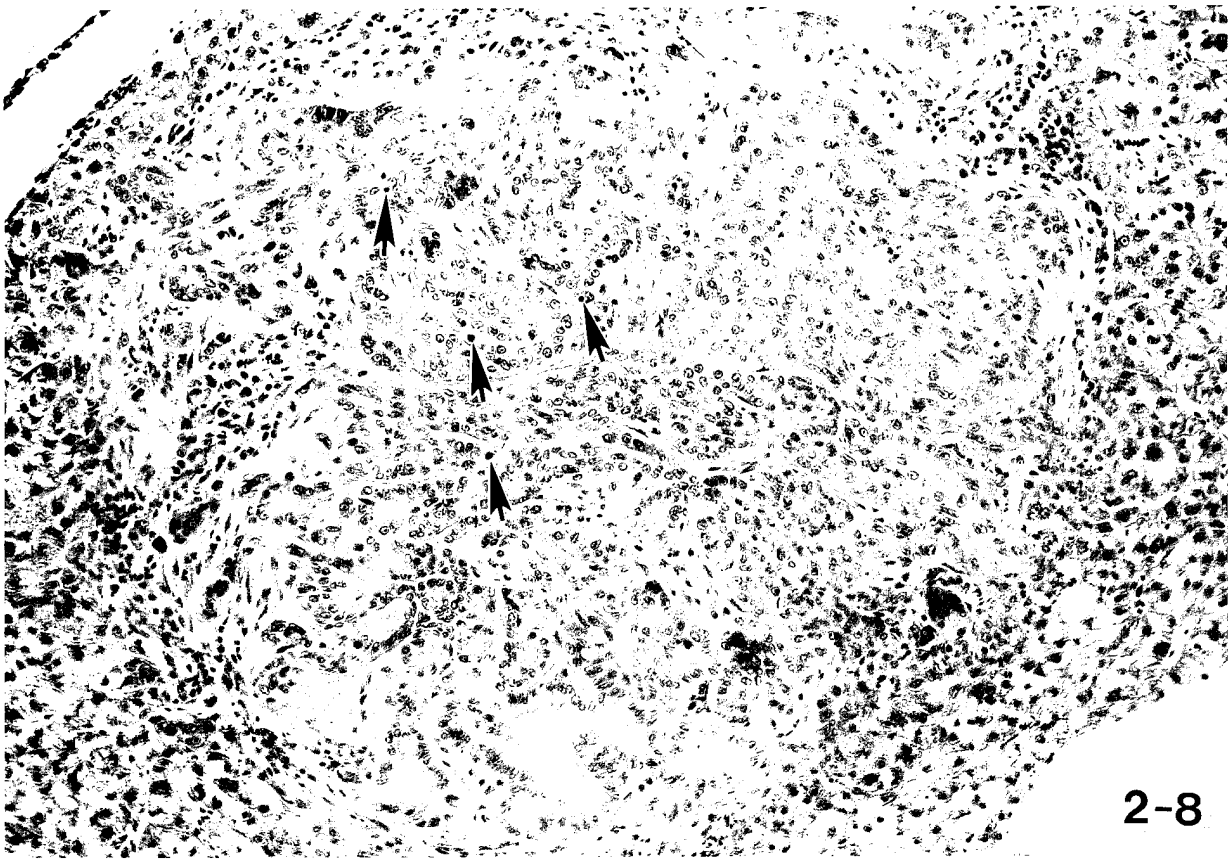


2-7

LEGENDS FOR FIGURES

Figure 2-8. Cholangiocarcinoma. Compare with fig. 2-7. Cells of carcinoma reveal a much higher incidence of pleomorphic nuclei. Cellular pattern is less like a duct and more solid, trabecular. Note the retention of duct-like structure at bottom of lesion. Cells within middle of lesion have proliferated until they appear as continuous sheets. At top left of field the lesion shows invasion of adjacent hepatic parenchyma. Arrows point to mitotic figures. Male medaka fed F/A diet before and after a 48 hr bath exposure to 350 ppm DEN. Fish was sampled four months after onset of exposure. Hematoxylin and eosin stain X 225.

Figure 2-9. Mixed hepato- and cholangio- cellular carcinoma. Large, spherical lesion contains both ductular and ductal resembling elements (cholangiocellular component). However, cells at the bottom of lesion and between ductlike structures resemble hepatocytes (arrows). Female medaka fed the PC-diet before and after a 48 hr bath exposure to 350 ppm DEN. Fish was sampled at three months after onset of exposure. Hematoxylin and eosin stain X 225.



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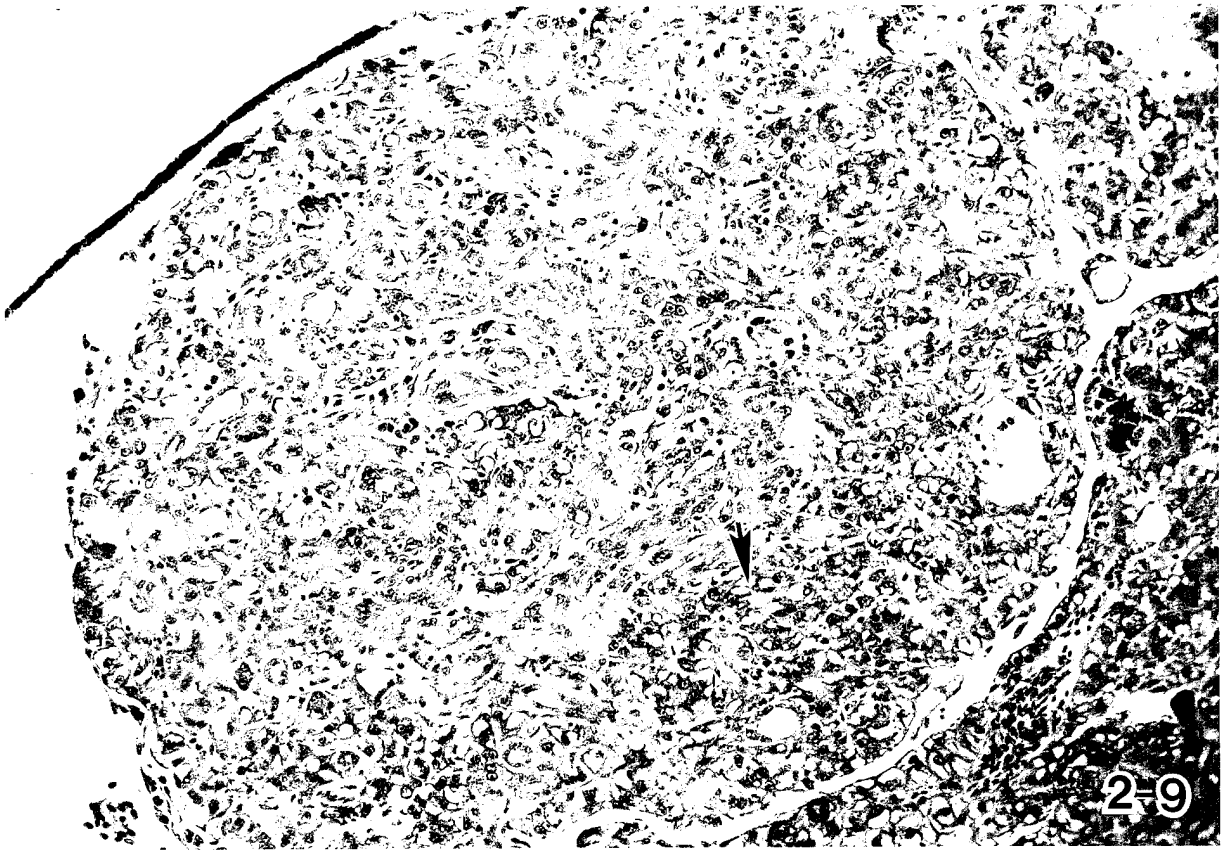

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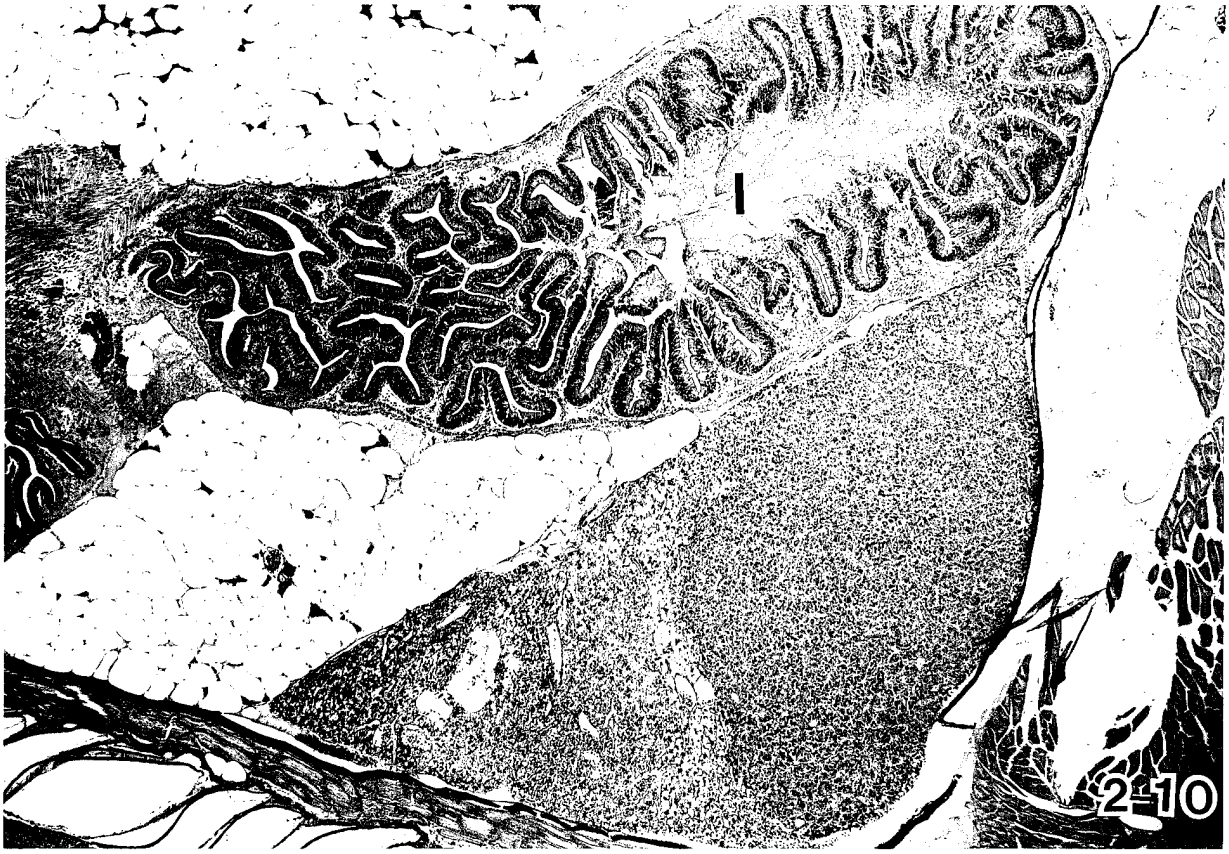

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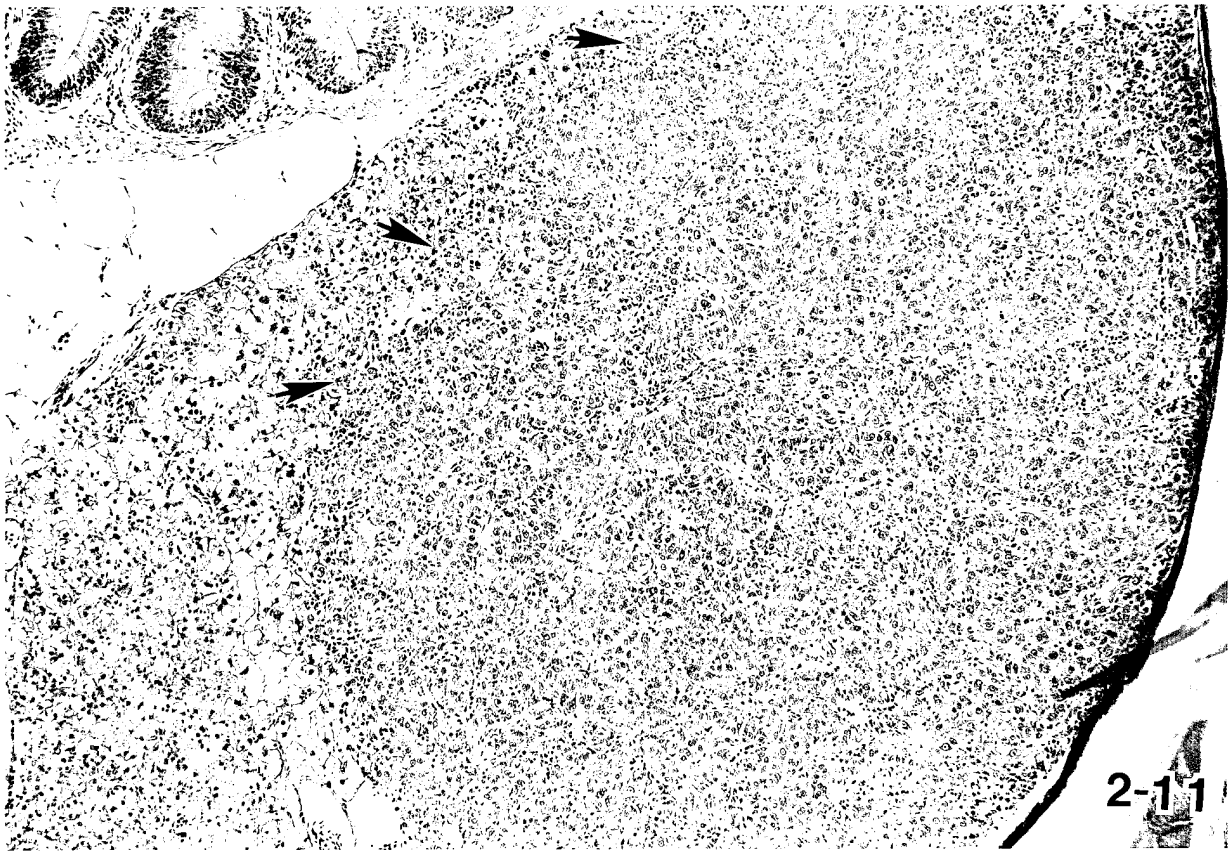

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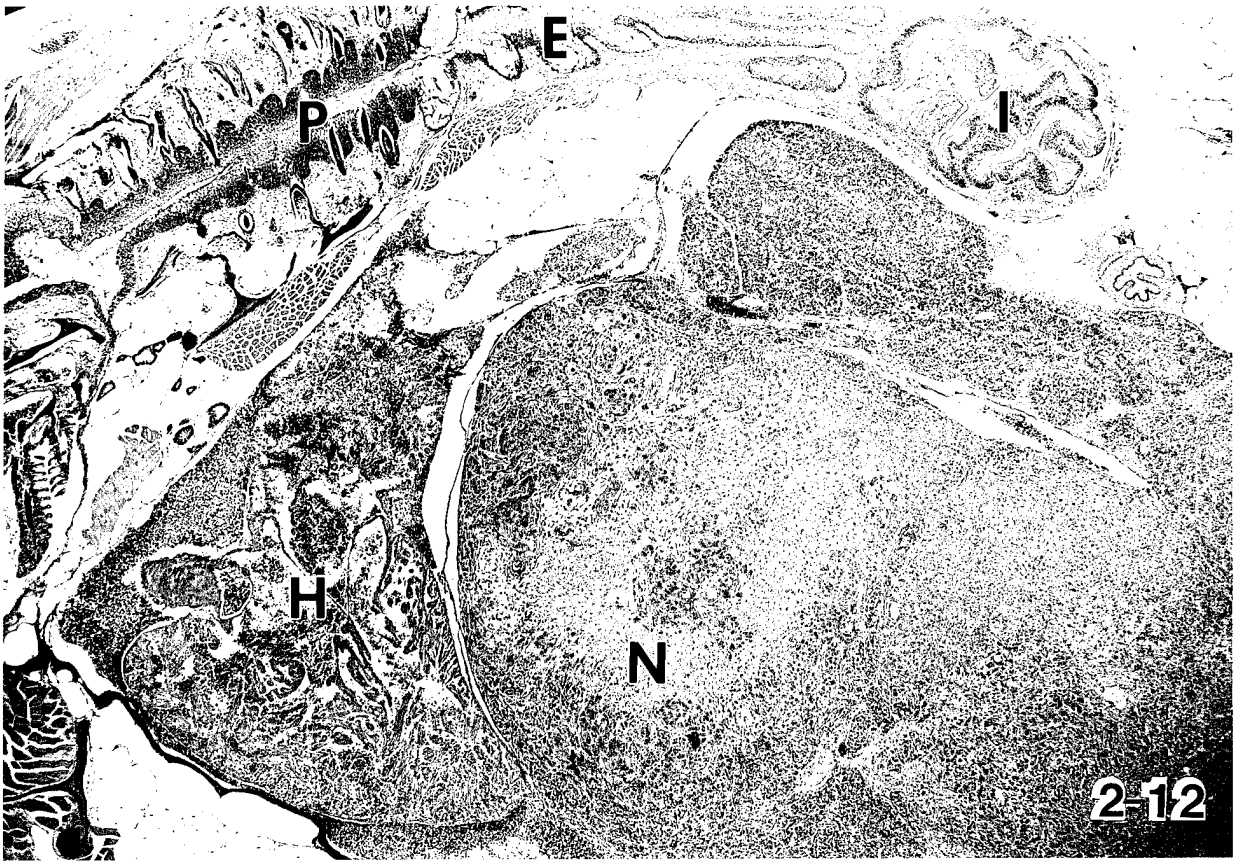
LEGENDS FOR FIGURES

Figure 2-12. Extremely large hepatocellular carcinoma occupying majority of liver section shows a central area of necrosis (N) and spongiosis hepatis (S). H = heart; E = Esophagus; P = Pharynx; I = Intestinal bulb. Female medaka sampled at seven months after onset of exposure to a bath of 350 ppm DEN for 48 hrs. Fish was fed F/A diet. Hematoxylin and eosin stain X 45.

Figure 2-13. Enlarged view of hepatocellular carcinoma shown in fig. 2-12. Note nuclear pleomorphism in tumor trabeculae. Necrotic areas (arrows) contrast with spongiosis hepatis (SH). Diet, sex, time of sampling and conditions of exposure are in legend to figure 2-12. Hematoxylin and eosin stain X 112.


E
P
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2-12

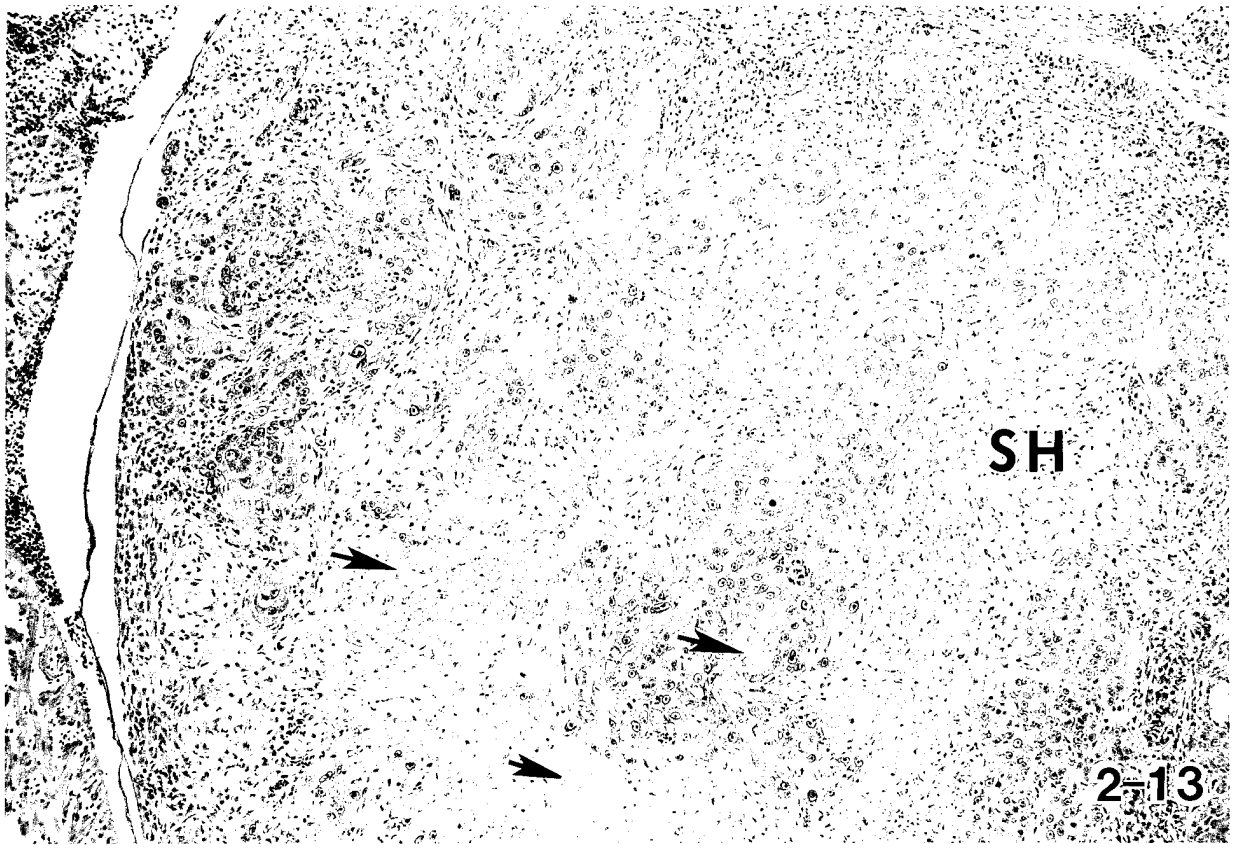
SH
2-13

Table 2-1. Den Exposure Mortality Data

	PC-diet		F/A Diet	
	Exposed	Control	Exposed	Control
4/29/91	0	0	0	0
4/30/91	1	0	1	0
5/l/91	0	0	0	0
PERCENT =	0.19%			

Table 2-2 DEN Post-Exposure Mortality Data

	PC-Diet			F/A-Diet	
	Exposed DEN #1	Exposed DEN #2	Control DEN #5	Exposed DEN #3	Exposed DEN #4
5/91	12	8	1	11	5
6/91	4	7	0	1	4
7/91	5	2	0	1	1
8/91	6	6	0	2	12
9/91	10	12	4	7	5
10/91	14	9	2	3	8
TOTAL	51	44	9	25	35
PERCENT	95 = 19%		9%	60 = 12%	

Table 2-3. Classification of liver alterations in hematoxylin and eosin-stained liver sections of medaka exposed to diethylnitrosamine.

FOCI OF CELLULAR ALTERATION	DESCRIPTION
General Features	
Size	Varies from small collection of cells to large lesions occupying 30% of liver sectional area. Often multiple. Occasionally mixed characteristics.
Border	Distinct. Cells not surrounded by capsule. Share architecture of surrounding parenchyma. One hepatic tubule may continue from adjacent (not involved parenchyma into the focus).
Architecture	Same as surrounding parenchyma.
Cytology	Nuclei usually normal but may be enlarged. Cytoplasm usually normal except for tinctorial properties.
Mitotic Figures	Data incomplete. Most foci don't reveal enhanced mitotic figures.
Specific Categories	
1. Basophilic	Normal to small hepatocytes with marked accentuation of cytoplasmic basophilia. Mitotic figures sometimes encountered.
A. Basophilic granular (Baso gr)	Cytoplasm granular and homogeneously basophilic.
B. Basophilic two-toned (Baso ++)	As above, especially around nucleus and at cell periphery. Cytoplasm also contains clear, pale stained areas thought to represent variable content of glycogen.

2. Eosinophilic	Individual hepatocytes vary in size. Some are quite large. Abnormally large nuclei may be present. Eosinophilia predominates over cytoplasm. Typical hepatocytes contain homogeneous pink cytoplasm.
A. Eosinophilic granular (EO grn)	Nuclei are usually non-remarkable. Cytoplasm is granular.
B. Eosinophilic hypertrophic (EO hyp)	Cells are markedly enlarged and may contain enlarged, atypical nuclei. Multiple nuclei may be present.
C. Eosinophilic proteinaceous (EO pro)	Cytoplasm shows ground-glass or hyalinized appearance. Nuclei as above.
3. Clear Cell	Cells of clear cell foci reveal little to no staining. Companion serial sections are positive for glycogen (PAS* method). Clear white nature of cytoplasm distinguishes these from other two foci. Margin of clear space is irregular as opposed to vacuolated cells (below).
A. Clear-basophilic (Cbas)	Cells are predominantly clear with basophilic peripheral cytoplasm.
B. Clear-eosinophilic (C eos)	Cells are predominantly clear with eosinophilic peripheral cytoplasm.
4. Vacuolated	Cytoplasm is vacuolated with normal nuclei. Vacuoles are generally due to lipid extraction during processing.
A. Vacuolated Focus - large (Vac lge)	Large vacuoles, 1-3, per cell.
B. Vacuolated Focus - small (Vac sm)	Small, multiple vacuoles, very numerous in each cell.
5. Mixed	These share features of the above.

A. Mixed foci	Cells may be of more than one of the above types. Predominant type is named first followed by other types (i.e., mixed basophilic and eosinophilic - basophilic predominant.
B. Amphophilic	Neither basophilic or eosinophilic but intermediate between the two and staining is enhanced.

NEOPLASMS

1. Hepatocellular Adenoma	Cells may stain basophilic, eosinophilic or a mixture. Lesion presents as a distinct nodular mass with well-defined margin. Component cells are arranged as tubules. Number of cells in an individual tubular profile usually increased.
2. Hepatocellular Carcinoma	No clear margin present. Growth extensions into adjacent tissue are usually seen. Gradations from minimal deviation (well-differentiated) to maximal deviation (poorly differentiated) are seen. Cells may appear as sheets. Nuclei are typically altered and often frequent in number.
3. Cholangioma (Biliary Adenoma)	Enlarged and hyperplastic profiles of tubular epithelium. Nuclei show piling up and slight gradation from normal appearance. Lesion is confined within basal lamina of biliary ducts/ductules.
4. Cholangiocarcinoma	Cells are anaplastic. Abundant nuclear and cytoplasmic atypia. Growth by extension into liver parenchyma and/or into intestine or head kidney. Mass may dissect along hepatic veins into pericardium.
5. Other Tumors	
A. Mixed	Carcinomas of both hepatocellular and cholangiocellular components. Both portions

	show anaplastic features.
B. Spindle Cell	Component cells are attenuated and arranged as strata. Tumor is thought to be of perisinusoidal fat-storing (Ito) cell origin.

*Periodic Acid Schiff's reagent method for glycogen with diastases as a histochemical control

Table 2-4

Effect of diet on frequency of liver neoplasms in medaka (*Oryzias latipes*) exposed to 350 ppm diethylnitrosamine for 48 hours

Months after Initiation	1	2	3	4	5	6	7
PC-diet[1]	0/20[3]	0/20	4/20	1/20	2/20	1/20	2/20
FL/A Diet[2]	0/20	0/20	2/20	2/20	5/20	3/20	7/20
Control							
PC-diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4
FL/A diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4

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[1]PC = Purified casein based diet. Fed from day 1 of hatch through day 21 and daily after 48 hour bath exposure.

[2]FL/A = Tetramin flake diet 5 days/wk. and 2 days/wk *Artemia* nauplii. Fed from day 1 of hatch through day 21 and daily after 48 hour bath exposure.

[3] Tumor bearing medaka per number exposed. Neoplasms included adenoma, cholangioma, hepatocellular carcinoma, cholangiocellular carcinoma, mixed hepato- and cholangio-cellular carcinoma, and spindle cell tumor.

Actual mean exposure concentration was 250.8 ppm (PC-diet fed group) and 257.4 ppm (flake *Artemia* fed group). Means included spectrophotometric assays at 12, 24, 36, and 48 hrs. Statistical analysis (Student's T test) revealed no significant differences between exposure concentrations ($P \leq 0.05$).

Chapter 3

Comparison of Hepatic Neoplasm Frequency in Medaka Fed a Purified Casein Versus Conventional Diet - Aflatoxin B_1

Introduction

Small aquarium fish such as the medaka (*Oryzias latipes*) have unique advantages as in vivo models for carcinogenesis research (Hoover et al., 1984). Briefly, their small body size permits thorough histologic examination at reduced cost, labor and time. Their small body size permits use of large numbers thereby improving statistical precision. Their low spontaneous tumor incidence permits examination of relationships of low concentrations of carcinogen to tumor formation. Current drawbacks have been lack of a companion data base for important relationships. These include: relationship of carcinogen concentration to DNA adduct formation and quantitative relationship of DNA adducts to tumor formation. By use of a refined diet, some of the interassay variation will be diminished and a stable benchmark to evaluate modulatory effects of environmental agents on the process can be achieved.

Unfortunately, the easily applied aqueous carcinogen, diethylnitrosamine, is by far the compound for which data on pathogenesis and quantitative aspects of FCA, adenomas and carcinomas is known. This small compound with a difficult to determine adduct, has made it difficult to establish genotoxic dosimetry and link that to tumor incidence. AFB_1 makes large adducts which may be quantified using existing technology. A drawback to the use of AFB_1 is the lack of an in vivo database for tumor incidence and the fact that regimes previously shown to be tumorigenic with small aquarium fishes involved prolonged dietary administration. Only two papers had previously been published on tumorigenesis in livers of small, aquarium species

exposed to aflatoxin (Sato et al., 1973; Hatanaka eta al., 1982). The Sato et al (1973) study fed 6 ppm AFB_1 to guppies (Lebistes reticulatus) while Hatanaka et al. (1982) used medaka fed 2.5 or 5 ppm AFB_1. In each of these only a few fish survived and the numbers of fish originally exposed were not given. Therefore, the toxicity and the number of animals dying prematurely were not known. Hatanaka et al. (1982) exposed medaka to 2.5 or to 5.0 ppm AFB_1 for six months in the diet. The total number of fish at each treatment level was not given. In the 25 survivors examined at the end of the six months exposure, 18 were reported to have "nodules". Whether these represented adenoma or carcinoma was not stated. In fish fed 5ppm for shorter duration, 26% showed nodules or more advanced lesions. Recent classifications of hepatic lesions in fish carcinogenesis bioassay refers to "nodules" as adenomas.

To determine the effect of diet on hepatic tumor formation in medaka after dietary exposure to AFB_1, we established the frequency of liver tinctorially altered foci (FCA), adenomas, cholangiomas, hepato-cellular carcinomas, cholangiocarcinomas, and mixed hepatocellular- and cholangiocellular-carcinomas and compared these in medaka fed either PC or F/A-diet before, during and after six months exposure to AFB_1.

Design of Study and Materials and Methods

A pilot study of 6 months duration and using dietary exposure to AFB_1 at one of four concentrations (0.0, 0.3, 1.0, and 3.0 ppm) was necessary to establish a concentration for the definitive study. The design used is described (Table 3-1). This range-finding study was done to enable us to select a concentration of the carcinogen which would result in appreciable incidence of hepatic neoplasms while permitting survival of a sufficient population of medaka for statistical significance. After the initial pilot study, a more definitive evaluation of the

suitability of the purified, casein-based ration for carcinogen bioassay work followed using a single concentration of AFB_1.

Table 3-1. Study Design

Diet[a]	AFB1 Concentration[b]	Duration
Purified Casein	0.0	6 months
	0.3	
	1.0	
	3.0	
Flake artemia	0.0	6 months
	0.3	
	1.0	
	3.0	

[a]All fish received respective diet from hatch until sexually mature (between 3 and 6 months) when carcinogen exposure was initiated.
[b] AFB_1 levels were verified by ELISA at 0, 3, and 6 months.

Fish culture and maintenance - Medaka (*Oryzias latipes*) eggs were collected from broodstock of golden variety medaka maintained at 25° C under a 16L:8D photoperiod. Fish were from broodstock obtained by crossing original medaka colony which was purchased from Carolina Biological Supply, supplemented periodically with fish from the Fort Detrick colony, with golden variety medaka imported from Japan in July 1989 (Nippon Goldfish, San Francisco, fish were from ponds in the vicinity of Yokohama, Japan). In practice, fertilized eggs from several females were pooled and incubated as described (Kirchen and West, 1976) without the presence of the antifungal agent, methylene blue. Eggs hatched in 9-10 days after fertilization.

Beginning on the day of hatch, fish were fed either a conventional flake diet for 5 days per

week and brine shrimp for the other 2 days per week (F/A-diet), or a casein based, purified ration (PC-diet). A sequential selection process which follows a standard U.S. Environmental Protection Agency procedure (Horning and Weber, 1985) was used to obtain fish for this study. A 25 cm wide net was used to trap and concentrate approximately 100 fish. From this common pool, while fish remained immersed, individuals were collected with a glass beaker and sequentially assigned to each of the aquaria. This process was repeated until the desired number of individuals was obtained in each aquarium. This process permits final selection of grossly healthy individuals and overcomes bias due to capture/evasion effect. To four 75-L acrylic aquaria (67 liters of water capacity), 350 larvae were assigned. This gave approximately 5.3 larvae per liter. Between 3 and 6 months of age, fish were transported to our carcinogen exposure facility. Six aquaria (pilot study), three for fish fed the conventional diet and three for fish fed the purified casein-based diet, were placed in a carcinogen-approved glove box. AFB_1 at three dietary levels (0.3, 1.0, or 3.0 AFB_1 was added to both diets and fed to respective medaka. These six aquaria were maintained as static exposures with over the side filters placed on each tank. Two additional aquaria were maintained on a laboratory bench in the exposure room. These housed fish which served as controls for each diet. Controls received diet free of AFB_1. Fish were fed twice daily. On the 2 days each week (Tuesday and Thursday), when the conventional diet fed fish were receiving brine shrimp, the PC fed fish received PC control diet free of AFB_1. The entire 6 months exposure was conducted inside glove boxes using a static renewal system. This less than optimal method of housing fish was dictated by environmental health and safety issues at that time. Fish were monitored twice daily and siphoning was employed to remove detritus and moribund or dead fish. A total of 25% of the water was

changed each week and more often if cloudy. Filter inserts (foam and carbon) were rinsed weekly and replaced monthly. At the end of the six months exposure period, survivors were transferred to our standard aquaria and permitted to grow for an additional three months.

In the definitive study, a system similar to our recirculating system used in culture facility, was employed in a room used only for the AFB_1.

Control diet preparation - The composition and preparation of the PC-diet for medaka is given in chapter 1. Dry purified ingredients, obtained from U.S. Biochemical Corporation (Cleveland, Ohio) and ICN Nutritional Biochemicals (Cleveland, Ohio) were mixed with a rotary mixer for 15 minutes. The oils were mixed with tert-butyl hydroquinone (TBHQ: Aldrich Chemicals, Milwaukee, WI) and mixed well into the dry ingredients. Distilled water was slowly added to form a slightly cohesive mixture. The PC-diet was then pressed through a stainless steel sieve (1.4 mm mesh size), freeze dried at -80°C in a Labconco freeze drier and stored under vacuum at -20°C.

AFB_1-containing diet preparation - Aflatoxin B_1 dissolved in acetone was added to the flaked portion of the commercial diet following Sato et al. (1973) and Hatanaka et al. (1982). Procedures included solubilization of aflatoxin in acetone, thorough mixing of the mixture of flake and acetone, and, final evaporation of the carrier solvent. A total three concentrations of aflatoxin B_1, on concentration per tank, was used. All fish in a given aquarium were fed only one level of aflatoxin B_1 concentration as follows: 0.0 (controls), 0.3, 1.0, and 3.0. Flakes to feed control fish were mixed with an identical amount of acetone and evaporated. No aflatoxin B_1 was added to the control diet. For the PC-diet, aflatoxin B_1, in acetone was mixed with the carbohydrate portion of the diet formulation. For the control (aflatoxin B_1 concentration = 0.0

ppm) only acetone was mixed with carbohydrate portion of diet. Following carrier evaporation, the carbohydrate component was mixed with the remaining dietary components (Table 1). A total of four concentrations of aflatoxin B_1, one concentration per tank was used. All fish in a given aquarium were fed only one level of aflatoxin B_1. Concentration (in ppm) was 0.0 (controls), 0.3, 1.0 and 3.0 Actual levels of aflatoxin in each diet were determined by gas chromatography in Dr. Dennis Hsieh's laboratory, Department of Environmental Toxicology at UC Davis. Both aflatoxin were proven palatable to medaka under conditions of the proposed test for up to three months. Histopathologic analysis indicated appearance of basophilic foci at 3 months after initiation of exposure in a preliminary pilot study. Acetone was selected as the carrier solvent shown by Dr. Hsieh not to alter aflatoxin structure. Fish were fed to slight excess twice daily and tanks were siphoned as needed to remove uneaten food and feces. The aflatoxin F/A-diet group was fed aflatoxin-spiked flakes 5 days/week and *Artemia nauplii* (with no aflatoxin) the remaining 2 days/week. PC-diet with aflatoxin was fed for 5 days/week. On days when FA fish received *Artemia nauplii*, and therefore no AFB_1, only clean PC-diet was fed all of the PC groups. Newly hatched brine shrimp were separated from unhatched and empty cysts and rinsed with reconstituted (EPA moderately hard) water before being fed to fish. Five fish were sampled from each tank for histopathologic analysis before initiation of aflatoxin feeding. Each aquarium was equipped with its own biological filtration system. Medaka exposed to AFB_1 were housed in aquaria within a carcinogen-approved hood. Control aquaria were maintained on a lab bench adjacent to the glove box. Room temperature was adjusted to equal that of the glove box ($25° \pm 1°C$). Partial replacement (25%) of the aquarium water was done weekly.

Water Quality - As described above, water quality was closely monitored during the length of the study. During the study, aquarium water was tested for possible leaching of AFB_1 from the diets. Actual measurements by gas chromatography have indicated no detectable AFB_1 in aquarium water after addition of diet. Carbon filtration was used in each tank, including the control, to help remove components not removed by routine cleaning procedures. The use of carbon filtration also insured removal of residual aflatoxin from exposure water. The aflatoxin regime was administered for 6 months. Color coding of diet types, tanks, and aflatoxin B_1 concentration was used to insure addition of correct diet for each tank. After this exposure period, fish were removed to clean recirculating water system for growout. They were placed in 75-L acrylic aquaria (72 liters of water) and all fish were fed aflatoxin-free FA- or PC-diet depending on their original designation. All fish fed FA- or PC-diet prior to and during exposure were returned to that respective diet without aflatoxin for growout. A subsample of 20 fish per aquarium (including controls) was taken immediately and again, three months after initiation of exposure, for wet weight determinations and general histopathology. A ten-inch net was used to concentrate fish and groups were isolated in glass beakers until 20 fish/tank were collected. This procedure decreases bias due to capture or evasion effects. The frequency of foci, adenomas and other neoplasms ($\pm$ per fish examined) among fish in each treatment was recorded by gender. At three months after termination of exposure, all remaining fish were sampled.

DNA concentration and purity- To determine DNA concentration and purity, two techniques were used. First, DNA was extracted from livers and purified using a protocol that required chloroform:isoamyl alcohol:phenol for extraction and hydroxyapatite for DNA purification. Once purified, the DNA was then hydrolyzed and analyzed by HPLC to determine

the number of AFB_1-diol adducts per million nucleotides. These were determined in pooled medaka livers after varying length of dietary exposure (in days) including: 31, 62, 180 and 215 using this methodology. Secondly, in studies designed to expand the database by exposing medaka to lower doses of AFB_1 over a short period of time, livers were removed, pooled, and stored frozen at -80°C. DNA was extracted using DNA STAT-60™, a single reagent for rapid DNA isolation (TEL-TEST "B", Inc. Friendswood, TX). DNA STAT-60™ is a single monophasic reagent containing a chaotropic cell disrupter and a non-corrosive, phenol-free extraction reagent. After homogenization in the monophasic reagent and addition of chloroform, the homogenate separates into aqueous and organic phases. The DNA remains in the aqueous phase or interface. The procedure takes approximately one-fourth the time of the first method. Another advantage is that many samples can be extracted simultaneously.

Livers from 20 medaka exposed to either 0.3- or to 1.0 ppm AFB_1 were removed after 10, 19, 30, 44, 54, 65 and 80 days of exposure, frozen and placed into tared 2ml clear microtubes (MH-820, Phenix Research Products) and the weights recorded. A total of 50-100 mg liver tissue was homogenized in 1 ml DNA STAT-60™ with a teflon rod. Homogenate was extracted twice with 0.2 ml chloroform per ml of DNA STAT-60™ and the tubes were shaken vigorously for 15 seconds. Material was stored at room temperature for 30 min. Homogenate was centrifuged at 12,000 g (13,000 rpm in SS-34 rotor) for 15 min at 4 °C. Following this, the DNA remained in the aqueous phase. The aqueous (upper) phase was transferred to a fresh microtube, re-extracted with chloroform, and centrifuged. The aqueous phase was transferred to a fresh microtube and an equal volume of isopropanol was added. The samples were mixed and stored at room temperature for 5-10 minutes. Samples were then centrifuged at 12,000 g for 10 min at 4°C.

After centrifugation, the DNA precipitate formed a small white pellet at the bottom of the tube.

Supernatant was removed and the DNA pellet was washed once with at least 1 ml of 75% ethanol per ml of the DNA STAT-60™ used for the final homogenization. Samples were vortex mixed and centrifuged at 7,500 g (10,500 rpm in SS-34 rotor) for 5 min at 4°C. After centrifugation, supernatant was removed and DNA pellet was air dried for 5-10 min. DNA pellets were then dissolved in 1.5 ml of HPLC water by vortex mixing or incubation for 10-15 min at 55-60°C.

To determine concentration and purity, samples (150 microliters volume) were transferred to an Ultra Micro quartz spectrophotometer cuvet (Sigma) and scanned using the HP UV/Vis Diode Array Spectrophotometer. A macro program was used to calculate DNA concentration and purity.

For acid hydrolysis, the purified DNA samples were transferred to 13 x 100 mm glass-stoppered test tubes. The tubes were placed in a beaker of boiling water for 2.5 hrs and after hydrolysis, cooled with cold running tap water.

AFB_1 DNA adduct determination - Prior to HPLC analysis, samples were further cleaned by passage over a C_{18} SEP-PAK. A 500 ml Erlenmeyer sidearm vacuum flask was connected to a SEP-PAK cartridge tip clamped to a ring stand and the columns were pre-washed with at least 2 column volumes of methanol followed by 2 column volumes of 0.05 N acetic acid under a light vacuum. The column was not allowed to dry during the procedure by rapid addition of second solvent wash prior to drying of surface of column adsorbent. After pre-washing, hydrolyzed samples were transferred to individual SEP-PAK columns. Each test tube was rinsed with 1-2 ml of 0.05 N acetic acid. Rinse was added to the column after the sample had passed

through the column. The column was filled to the top with 0.05 N acetic acid and subsequently rinsed with two more column volumes of acetic acid. After the final acetic acid rinse had passed through the column, the vacuum was increased until water was no longer observed coming out of the column. The vacuum was then turned off and the vacuum line disconnected from the column tip.

AFB_1-diol was eluted from the SEP-PAK column with 3 ml of methanol and collected into 4 ml amber vials fitted with teflon caps. The methanol eluates were stored in a refrigerator overnight and on the next day, 1.86 ml portions were removed and evaporated to dryness under a gentle stream of nitrogen. These samples were then resuspended in 0.08 ml of HPLC mobile phase (acetic acid/acetonitrile, 70:30) and analyzed by HPLC using a Waters system fitted with a Waters Novapak C_{18} column (3.9 x 300 column, mobile phase: 70% 0.05 N acetic acid/30% acetonitrile, flow rate: 0.75 ml/min), and a Waters 420 -AC fluorescence detector.

Prior to sample analysis, 0.010 ml of each AFB_1-diol standard was injected twice and a standard curve was constructed by plotting peak area vs. picograms of AFB_1-diol injected. For each sample, 0.020 ml was injected on the HPLC and analyzed for AFB_1-diol. The amount of AFB_1-diol in each sample was determined by measuring the peak area and calculating the picograms of AFB_1-diol from the standard curve.

Histopathologic analysis - Because there are no published guidelines for nomenclature of fish, specifically medaka, hepatic neoplasms and associated lesions, we adopted aspects of uniform criteria from rodent bioassay descriptions and fit them to our medaka observations (Chapter 2). The adoption of such criteria, while facilitating uniformity of bioassay results, must reflect the breadth and nature of histopathologic alterations in medaka. Lesions included foci and

neoplasms. Only those lesions visible with hematoxylin and eosin (H&E) staining were enumerated. The aflatoxin B_1 pilot study was designed as a preliminary experiment to determine the concentration of this component in the diet that provides sufficient foci or neoplastic lesion frequency. Also, since the duration of growout necessary for full expression of neoplastic potential was unknown, we were interested in determining the time to tumor. For the above reasons, we included foci as an additional endpoint. Each liver section from an individual fish will have a focus or neoplasm or be free of one or the other. Here, the Chi-square statistic was used to compare specific lesion frequencies in control aflatoxin-exposed dietary groups using a 2X contingency table. The expected lesion frequency was the frequency of control fish with either foci (separately) or neoplasms (after arcsin transformation).

At necropsy for scheduled samplings, medaka were fixed by immersion in Bouin's fixative and injected with fixative in peritoneal cavity. The next day, carcasses were sectioned in as near the sagittal plane as possible and the two halves were placed in neutral buffered formalin (10%) and cassetted for further fixation, dehydrated by passage through a graded ethanol series, and cleared in xylene. Each half was embedded cut surface down and after paraffin blocks had hardened they were sectioned with a rotary microtome at 5-7 microns thickness. Step sections separated by 100 microns were made until major viscera were represented.

Each fish was classified by gonadal histology as male, female or not present. **Autolysis**- the extent was analyzed primarily based on appearance of the intestinal tract, and graded as: 1) minimal, with all cell membranes intact; 2) mild, a few cells on the tips of the villi were affected; 3) moderate, at least one section of intestine had transmural autolysis; or 4) severe, for more than focal transmural autolysis. Mild or moderate autolysis sometimes occurred in fish that were

placed in fixative at death, primarily as a result of poor penetration of the fixative in the region of the gall bladder. By comparison, severe autolysis occurred only in fish that were found dead and placed in fixative more than an hour after death.

Hepatic glycogen - was identified by clear, poorly demarcated, irregular, cytoplasmic vacuoles. The extent of glycogen was ranked and scored as: 1) minimum, no obvious hepatocellular vacuoles; 2) moderate, area of hepatocellular vacuoles was less than or equal to nuclear area; or 3) abundant, area of hepatocellular vacuoles greater than nuclear area. In general, hepatocellular glycogen seemed related to general condition of fish; healthy feeding fish had more glycogen area than did unhealthy fish. In the pilot study, glycogen stores increased among all AFB_1 groups after termination of exposure.

Disseminated mycobacteriosis (DM) - *Mycobacterium avium* was cultured in our laboratory in 1990 and 1991. *Mycobacterium chelonei* was cultured in 1991 and 1992. *Mycobacterium fortuitum* and *Mycobacterium marinum* were cultured from the gravel and water of aquaria maintained in our histopathology laboratory in 1987 - 1988, but the latter two organisms were not cultured directly from medaka. We have consulted bacteriologists and the veterinary staff in our UC Davis animal care facility and have the following reasons why we have encountered this problem in our cultures. First, mycobacteria are common soil and water bacteria that invade immunocompromised hosts more readily than healthy hosts (for example, disseminated *M. avium* is a major cause of death in human patients with acquired immunodeficiency syndrome but it rarely infects the general population). Secondly, in our broodstock fish, mycobacteriosis is rare in fish less than 200 days old. In our exposed fish, incidence of mycobacteriosis was as high as 70% in 9-month old fish reared in carcinogen exposure facility. Our general feeling is that

mycobacterium infection is a general indicator of medaka health and immune function. Severity and incidence generally increase with exposure to toxic compounds, overcrowding and handling. Decreased incidence of mycobacteriosis in AFB_1-exposed fish 3 months after termination of exposure might be related more to decreased density of high-exposure tanks than to previous exposure.

Mycobacterial organisms within phagocyte cytoplasm had a distinctive pale-basophilic fibrillar appearance. Staining with acid-fast stains was variable and depended on the type of fixative and time in fixative before processing.

Scoring was 0- when no *Mycobacterium* organisms were present; 1- when *Mycobacterium* organisms were limited to a small area of one organ; 2- when *Mycobacterium* organisms were present and disseminated to more than one organ but not resulting in distension of an organ; 3- *Mycobacteria* were disseminated in more than one organ and significantly distended one organ; and 4- *Mycobacteria* were disseminated in more than one organ and significantly distended more than one organ.

Atrial Phagocyte Hypertrophy(APH) - is probably a combination of at least two processes (and may also include any myocardial cell). The following are characteristics of this lesion: 1) accumulation of yellow-brown or gray-black pigment that distends phagocyte cytoplasm. In moderate to severe cases, pigment-laden cells pile up on top of each other to form nodules. Unlike renal or hepatic macrophage aggregates, APH scores included *Mycobacterium* - filled phagocytes. 2) Cytoplasmic hypertrophy was a prominent feature of the affected cells. Normal atrial lining cells have condensed nuclei and little or no visible cytoplasm on routine H&E sections. By comparison, hypertrophied cells had round nuclei and the expanded eosinophilic

cytoplasm resulted in cuboidal shaped cells which never were seen to pile up on each other. Scoring: 0- no pigment and no hypertrophy; 1- section of heart showed fewer than 3-4 pigmented nodules and no cuboidal phagocytes; 2- heart had more than 4 pigment-laden phagocytes, or at least some of the phagocytes had swollen cytoplasm; 3-phagocytes were occasionally piled up to form nodules; and 4- nodules were large enough to restrict blood flow.

Vacuolar Encephalopathy (VE) - clear, round, well-demarcated vacuoles were scattered throughout the brain but were most prominent in the optic lobe. The cellular location of the vacuoles has not been determined, but they might be distended capillaries. Vacuoles are probably artifacts of fixation and processing. Scoring: 0- for $\leq$1 vacuole per brain section; 1- for 2-6 vacuoles per brain section; 2- for 7-30 vacuoles per brain section; 3- for 31-100 vacuoles per brain section; 4- > 100 vacuoles per brain section.

Spongiosis Hepatis (SH) - the hepatic parenchyma sometimes had roughly spherical foci in which hepatocytes were absent but the mesenchymal network was retained, resulting in a net-like appearance. Foci of spongiosis hepatis were often centered around bile ductules. Scoring: 0- for no spongiotic foci; 1- one small spongiotic focus in the liver section; 2- for one large or up to three small spongiotic foci; 3- for 3-7 spongiotic foci in the section; and 4- for >7 spongiotic foci per section.

Hepatic Macrophage Aggregates (HMA) - aggregates of macrophages included in this lesion category had pale yellow-brown pigment. Note that *Mycobacterium*-filled macrophages were not included in this lesion score, but were included with the **DM** (above). Scoring: 0- no macrophage aggregates; 1- macrophage aggregates, usually <5 per section, were $\leq$40 um in diameter; examination with the 40X objective lens was required to find aggregates scored as "1";

2- ≥3 to 7 aggregates per section, with at least one aggregate > 40 µm in diameter; examination with the 10X objective was sufficient to find aggregates scored as "2"; 3- for >7 macrophage aggregates but total aggregate area was less than total area of hepatocytes; 4- for livers with total macrophage area greater than total area of hepatocytes.

Renal Macrophage Aggregates (RMA) - aggregates of macrophages included in this lesion category had pale yellow-brown pigment. Note that *Mycobacterium*-filled macrophages were not included in this lesion score but were included in the **DM** score. Scoring: 0- no macrophage aggregates; 1- macrophage aggregates, usually <5 per kidney section and ≤ 40 µm in diameter; examination with the 40X objective was necessary to find aggregates scored as "1"; 2- ≥3 to 7 macrophage aggregates per section with at least one aggregate >40 µm in diameter; examination with 10X objective lens was sufficient to find aggregates scored as "2"; 3- > 7 macrophage aggregates with several > 40 µm in diameter but total aggregate area was less than that of total kidney parenchymal area; and 4- kidneys in which total macrophage area exceeded that of the kidney parenchymal area.

Gas Gland Hyperplasia (GGH) - scoring of this lesion was based on the relative thickness of the epithelium of the gas gland. The gas gland is normally a thin crescent of cuboidal to low-columnar eosinophilic cells at the anterior margin of the swimbladder. Scoring: 0- gas gland epithelium was ≤3 cell layers thick; 1- gas gland epithelium >3 but ≤5 cell layers thick; 2- gas gland epithelium >5 but ≤9 cell layers thick; 3- gas gland epithelium is >9 cell layers thick but retains a crescentic shape; and 4- hyperplastic gas gland epithelium bulges to form a mass broader than a hemisphere.

Definitive AFB_1 study materials and methods - Prior to initiation of the definitive AFB_1, bioassay, a closed recirculating system with four, 75-liter volume tanks was built. To establish the function of the biological filter, 20 fish were housed in each tank and ammonia and nitrite levels were monitored during this period to ascertain the nitrifying potential of the filters for the recirculating system. Upon verification of function, the experimental fish were then moved to each tank and allowed to acclimate. Water chemistry was performed on all tanks for the first 12 days, then weekly for the duration of the study. Details of acceptable ranges for water quality values are found in Chapter 1 of this report. Detailed aspects of the fixation, embedment, and processing of fish for paraffin section analysis are contained within Chapter 2 of this report. All procedures performed in definitive study were identical to those described in pilot study and/or Chapter 2 above.

RESULTS

AFB_1 DNA Adducts (ADA) - Data on ADA in medaka consuming diet containing 3ppm AFB_1 are shown in Table 3-2. After 31 days of exposure at the 3-ppm level, aflatoxin DNA adducts were present at 5.24 µmol AFB_1/mol DNA. This appeared to be a steady-state concentration since at 62 days, the level was approximately the same, and, at 180 days, at the termination of the exposure, was 5.01. In one group evaluated 35 days after the exposure was terminated, the concentration had decreased to approximately 2 ADA.

Table 3-2. AFB_1-DNA Adduct (ADA) Data for the 3 ppm Liver Samples

Exposure Duration (days)	ADA (µmol AFB/mol DNA
0	0
31	5.24
62	4.50
180	5.01
35 days after exposure	1.96

One goal of the AFB_1 exposure study was to determine the steady-state ADA in medaka liver as a function of the concentration of AFB_1 in the diet. The 3 ppm data indicated that a steady-state is reached after approximately 31 days. Since data existed at this time for only the 3 ppm dietary concentration, we decided to expand the database by determining ADA in medaka exposed to lower concentrations over a shorter period of time.

Data on liver weights, DNA recovered, micrograms of DNA per milligram of liver, optical density ratio of absorbance at 260 divided by absorbance at 280 and DNA concentration in medaka consuming diet containing 0.30- or 1.0 ppm AFB_1 for 10, 19, 30, 44, 54, 65, or 80 days

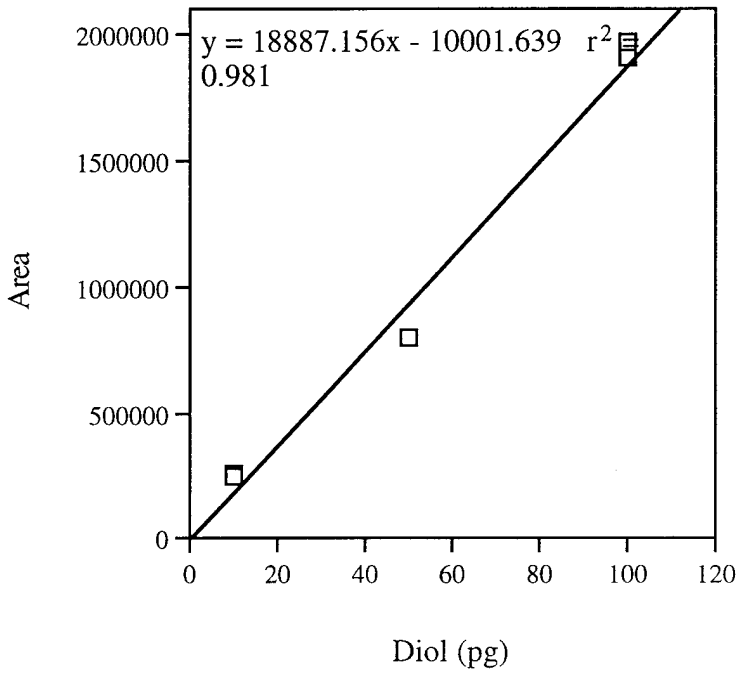
are shown in Table 3-3.

Table 3-3. Data on Medaka Livers Extracted with DNA Stat 60.

Sample I.D.	Liver Weight (mg)	DNA Recovered (µ/g)	µg DNA/ mg Liver	OD 260/280	DNA Conc (ng/µl
0.3 ppm AFB1					
day 10	36.40	80.75	2.22	1.64	53.83
day 19	35.30	137.57	3.89	1.68	91.71
day 30	61.60	115.01	1.87	1.67	76.67
day 44	105.10	191.82	1.83	1.67	127.88
day 54	94.60	195.98	2.07	1.59	130.65
day 65	44.80	99.69	2.22	1.71	66.46
day 80	120.20	120.72	1.00	1.72	80.48
1.0 ppm AFB1					
day 10	85.10	104.73	1.23	1.57	69.82
day 30	59.60	129.29	2.17	1.57	86.19
day 44	129.30	143.63	1.11	1.58	95.75
day 54	118.80	117.36	0.99	1.50	78.24
day 65	111.90	123.05	1.10	1.54	82.03
day 80	217.70	152.66	0.70	1.60	101.77

Prior to HPLC analysis, a standard curve was constructed by plotting peak area vs. picograms of AFB_1-diol injected as shown in Figure 3-1. The detection limit was 10 picograms of AFB_1-diol.

Figure 3-1. Aflatoxin B_1-Diol HPLC Standard Curve.



For each sample, 0.020 ml was injected on the HPLC and analyzed for ABF_1-diol.

HPLC analysis of livers from medaka exposed for varying time periods to 0.3 ppm AFB_1 are shown in Table 3-4.

Table 3-4. HPLC Analysis of Medaka Livers Extracted with DNA STAT-60

0.3 ppm AFB1	Vol inj (µl)	DNA inj (µg)	Area Peak	Diol inj (pg)	Correct. Diol (pg)*		ADA/MN
day 10	20	12.52	482290	26.06	45.75		3.49
		12.52	672180	36.12	63.40		4.83
		12.52	580250	31.25	54.86		4.18
						Avg	**4.16**
						Std Dev	**0.67**
day 19	20	21.32	418470	22.69	39.82		1.78
		21.32	293640	16.08	28.22		1.26
		21.32	181160	10.12	17.77		0.79
						Avg	**1.28**
						Std Dev	**0.49**
day 30	20	17.83	183570	10.25	17.99		0.96
		17.83	426600	23.12	40.58		2.17
		17.83	325250	17.75	31.16		1.67
						Avg	**1.60**
						Std Dev	**0.61**
day 44	20	29.73	474410	25.65	45.02		1.44
		29.73	421280	22.83	40.08		1.29
		29.73	122890	7.04	12.35		0.40
						Avg	**1.04**
						Std Dev	**0.56**
day 54	20	30.38	352090	19.17	33.65		1.06
		30.38	228280	12.62	22.15		0.70
		30.38	358050	19.49	34.21		1.07
						Avg	**0.94**
						Std Dev	**0.21**

Table 3-4. HPLC Analysis of Medaka Livers Extracted with DNA STAT-60

0.3 ppm AFB1	Vol inj (µl)	DNA inj (µg)	Area Peak	Diol inj (pg)	Correct. Diol (pg)*		ADA/MN
day 65	20	15.45	56974	3.55	6.22		0.38
		15.45	134710	7.66	13.45		0.83
		15.45	69818	4.23	7.42		0.46
						Avg	**0.56**
						Std Dev	**0.24**
day 80	20	18.71	197140	10.97	19.25		0.98
		18.71	244690	13.48	23.67		1.21
		18.71	52530	3.31	5.81		0.30
						Avg	**0.83**
						Std Dev	**0.47**

*Data corrected for 63.3% hydrolysis efficiency and 90% SepPak recovery

HPLC analysis of livers from medaka exposed for varying time periods to 1.0 ppm AFB_1 are shown in Table 3-5.

Table 3-5. HPLC Analysis of Medaka Livers Extracted with DNA Stat-60.

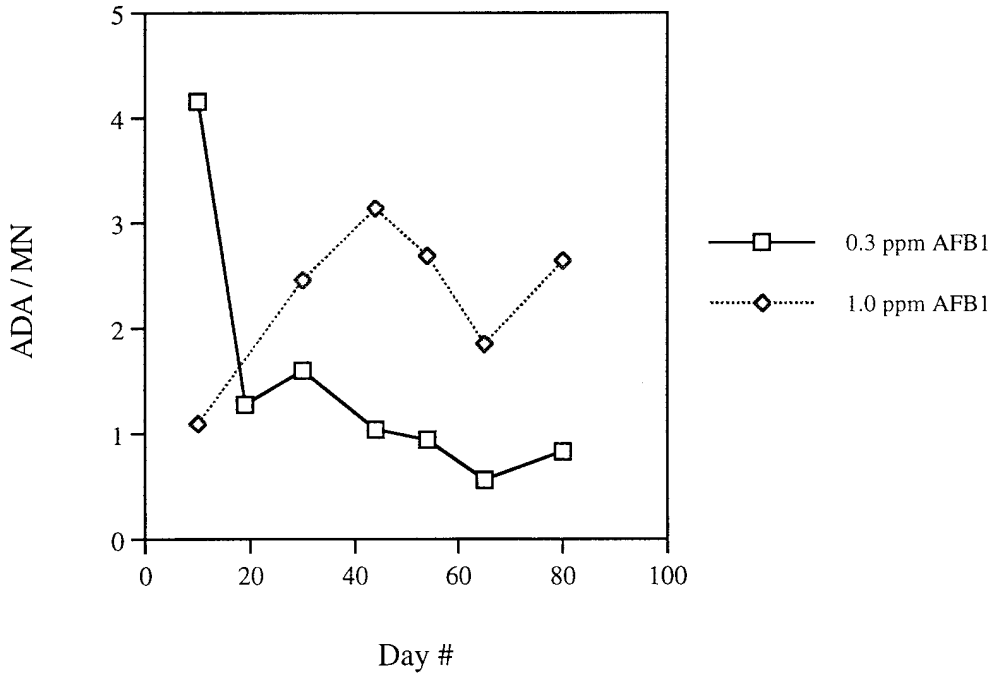
0.3 ppm AFB1	Vol inj (µl)	DNA inj (µg)	Area Peak	Diol inj (pg)	Correct. Diol (pg)*		ADA/MN
day 10	20	16.23	117900	6.77	11.89		0.70
		16.23	137540	7.81	13.71		0.81
		16.23	310690	16.98	29.80		1.75
						Avg	**1.09**
						Std Dev	**0.58**
day 30	20	20.04	748700	40.17	70.51		3.36
		20.04	732150	39.29	68.97		3.28
		20.04	156980	8.84	15.52		0.74
						Avg	**2.46**
						Std Dev	**1.49**
day 44	20	22.26	715330	38.40	67.41		2.89
		22.26	843280	45.18	79.30		3.40
		22.26	775190	41.57	72.97		3.13
						Avg	**3.14**
						Std Dev	**0.25**
day 54	20	18.19	679170	36.49	64.05		3.36
		18.19	485190	26.22	46.02		2.41
		18.19	464060	25.10	44.06		2.31
						Avg	**2.69**
						Std Dev	**0.58**
day 65	20	19.07	278140	15.26	26.78		1.34
		19.07	422280	22.89	40.17		2.01
		19.07	463060	25.05	43.96		2.20
						Avg	**1.85**
						Std Dev	**0.45**

Table 3-5. HPLC Analysis of Medaka Livers Extracted with DNA STAT-60.							
0.3 ppm AFB1	**Vol inj (µl)**	**DNA inj (µg)**	**Area Peak**	**Diol inj (pg)**	**Correct. Diol (pg)***		**ADA/MN**
day 80	20	23.66	587580	31.64	55.54		2.24
		23.66	646780	34.77	61.04		2.46
		23.66	847440	45.40	79.69		3.21
						Avg	**2.64**
						Std Dev	**0.51**

*Data corrected for 63.3% hydrolysis efficiency and 90% SepPak recovery

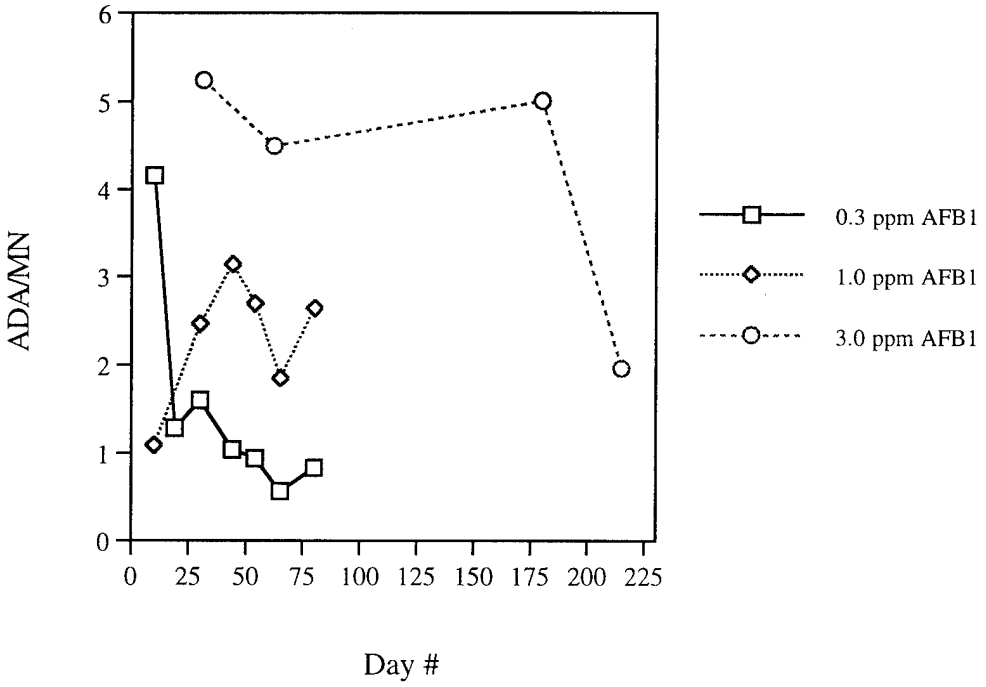
The data from Tables 3-4 and 3-5 are summarized in Figure 3-2. and represent the number of ADA per million nucleotides at two concentrations of AFB_1 over a feeding period of 80 days. Each time point represents the average of three separate HPLC injections.

Figure 3-2. ADA vs. Time for 2 different AFB_1 Doses.



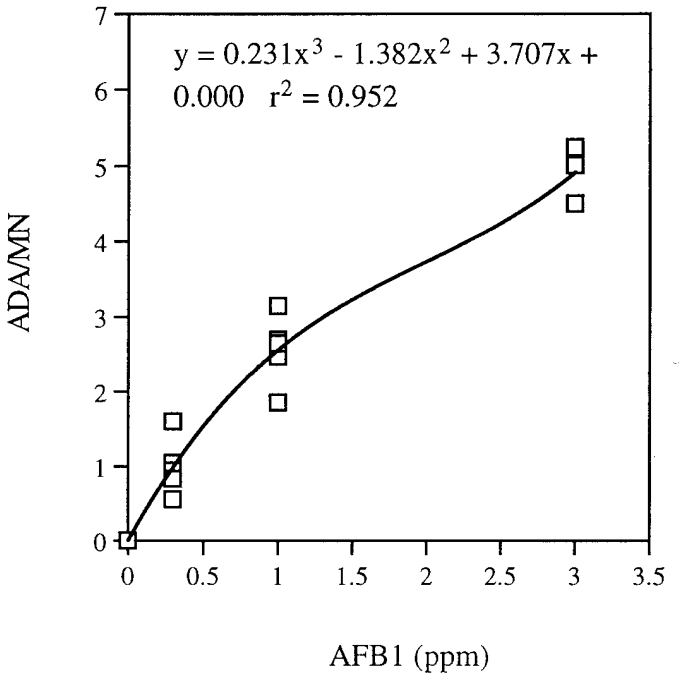
The number of ADA per million nucleotides has been determined for 3.0 ppm AFB_1 in the diet over a feeding period of 180 days (Table 3-2). This data has been combined with the data for 0.3 and 1.0 ppm AFB_1 and is shown for comparison in Figure 3-3.

Figure 3-3. ADA vs. Time for 3 different AFB_1 Doses.



Assuming that a steady state level of ADA is reached between 30 and 80 days of exposure, plotting the range of ADA values for each dose of AFB_1 over this time period results in a dose-response curve for ADA vs. AFB_1 in the medaka diet as shown in Figure 3-4.

Figure 3-4. Dose-Response Curve for AFB_1.



In conclusion, our experience for AFB_1 and DNA adducts indicate that for 0.3 ppm and 1.0 ppm AFB_1, the highest numbers of ADA/MN are observed at 30 and 44 days respectively. The relatively high number of ADA/MN for the 0.3 ppm AFB_1 Day 10 sample is unexplained. This point would be expected to follow the dose-response trend observed for the 1ppm AFB_1 time points and to lay at or below 1.0 ADA/MN. Assuming that a steady-level of ADA is reached between 30 and 80 days of exposure to either 0.3-, 1.0- or 3.0 ppm AFB_1 in the diet and the

number of ADA/MN observed. Also, the DNA STAT-60™ procedure saves a significant amount of time in extracting and purifying DNA from medaka liver tissue when compared to the previous method requiring the use and disposal of phenol. In addition, multiple samples can be extracted simultaneously.

Problems encountered - Physical restrictions were involved in this pilot aflatoxin dietary study. First, exposure was static and over a prolonged (6 months duration). Housing space within carcinogen approved glove boxes was limited and required that we use the static exposures. The present aflatoxin B_1 study was undertaken as a pilot project. The numbers of fish at each concentration level were maintained in order to derive useful information. We use "pilot" since the maximum tolerated concentration of aflatoxin is not known for medaka. After the pilot study, a repeated exposure at a single concentration was used (definitive study), thereby insuring numbers necessary to derive statistically significant information on tumor frequency as a function of diet. Duration of growout, after the 6-month exposure was an unknown and needed to be determined. Finally, the single aflatoxin level at which appropriate frequencies of tumors can be achieved was determined. Data from this initial pilot study was used to formulate the final design. The results presented for the pilot study should be regarded as preliminary in nature but when coupled with the definitive study shoe a more complete picture.

Histopathology results - six months sampling; pilot study - Findings are presented in both histogram and tabular form. As labels indicate, all data used to formulate histograms is presented in appendices (Appendix 1 for 6 months sampling; Appendix 2 for 9 months sampling). What follows is first from the six months sampling followed by the nine months sampling.

Using the histopathology alterations (lesions) defined in the methods (above) we scored for

severity (see methods) and a histogram of the lesions encountered in male and female control medaka after 6 months of static renewal culture are shown for each diet (Fig. 3-5). Areas of the hepatocyte cytoplasm corresponding to glycogen depots (HG) were higher in males and females fed the F/A-diet. Lowest value for this parameter was seen in PC-fed males, and indicates that glycogen was more depleted in these medaka. For disseminated mycobacteriosis (DM), lower mean scores were seen with F/A-diet and females showed lower scores for this condition. The PC-fed male medaka scored higher than their FA-fed counterparts. The condition, atrial phagocyte hypertrophy (APH), females scored higher than their male counterparts and females fed the PC-diet were highest. The condition, spongiosis hepatis (SH), was higher in FA-fed medaka and at a similar level for each gender. Score was low in females fed PC-diet and SH was not present in their male counterparts (Fig. 3-5). Gas gland hyperplasia (GGH) scores were higher in PC- fed medaka than in their FA-fed cohorts. Males fed F/A-diet showed most variation in scores, but females of PC-fed fish showed highest mean scores.

In table 3-6, we present findings of the histopathologic analysis of control medaka by diet and gender. Of the FA-fed medaka (56 females and 49 males), one male showed the presence of alternating focal lesions which stained with mixed basophilic and eosinophilic cells. Similarly, only one medaka fed the PC control diet showed alternating lesions with mixed basophilic and eosinophilic cells. At very low frequency, medaka fed the control F/A-diet showed foci of cellular alteration (FCA). Some of these phenotypes are those of cells within eventual hepatic neoplasms; others, foamy and vacuolated foci, have been seen in older control animals but are apparently not associated directly with hepatic neoplasms. Note FCA frequency in FA fed medaka was 0.2 and 0.3 in male and female, respectively, compared in identical sequence to 0.16

and 0.07 in PC-fed medaka. Medaka consuming control F/A-diet showed two neoplasms, one for each gender. A single neoplasm was found in one female consuming control PC-diet.

In the six month sampling, when body weights of medaka were determined for each group, it is obvious that for the controls, and AFB_1 dietary groups of 0.3 ppm, and 3.0 ppm, that PC fed fish outweighed their FA fed counterparts. Results in both dietary groups were similar at the 1.0 ppm concentration (Fig. 3-6).

Figure 3-5. Mean severity scores for lesions appearing in livers of control medaka. Six month sampling after maintenance under static renewal conditions of the pilot study. HG = hepatocellular glycogen; DM = disseminated mycobacteriosis; APH = atrial phagocytic hypertrophy; SH = spongiosis hepatis; GGH = gas gland hyperplasia.

Numbers for Histogram Graphics:

	Diet	**Stat.**	**HG**	**DM**	**APH**	**SH**	**GGH**	**freq**
Female	FA	N	56	56	52	56	12	56
		mean	1.9	1	1.6	.82	.92	11
		± SE	.12	.14	.11	.14	.29	.20
Male	FA	N	44	49	37	44	4	42
		mean	1.8	1.4	1.4	.89	1.3	11
		± SE	.13	.16	.13	.17	.75	.26
Female	PC	N	25	25	22	24	4	25
		mean	1.6	1.2	2.4	.21	2.5	4
		± SE	.16	.29	.11	.15	.29	.16
Male	PC	N	15	18	16	16	8	15
		mean	1.5	1.9	1.9	.06	2.1	1
		± SE	1.9	.39	.22	.06	.13	.07

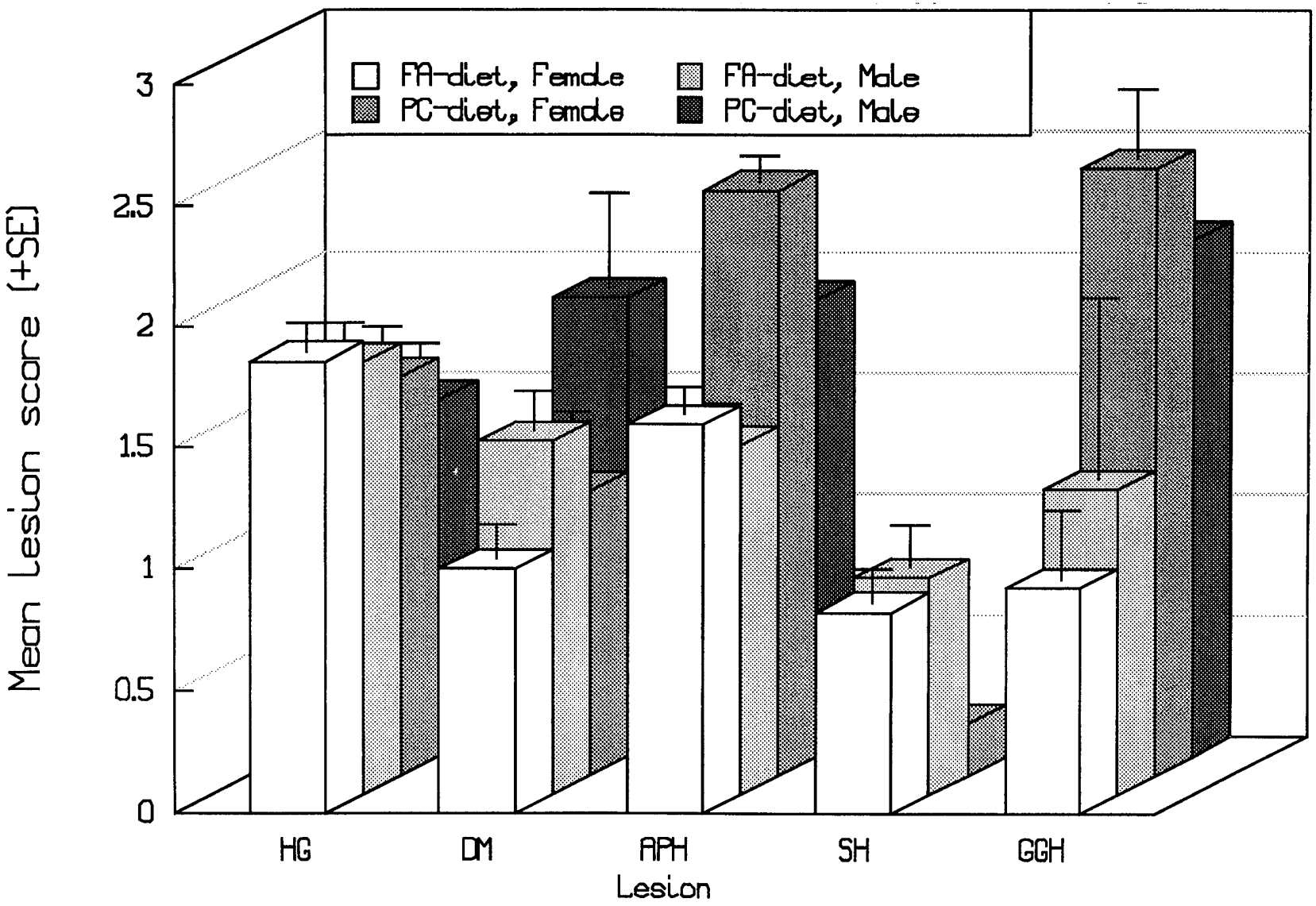

FA-diet, Female
PC-diet, Female
FA-diet, Male
PC-diet, Male
Mean Lesion score (+SE)
0
0.5
1
1.5
2
2.5
3
HG
DM
APH
SH
GGH
Lesion

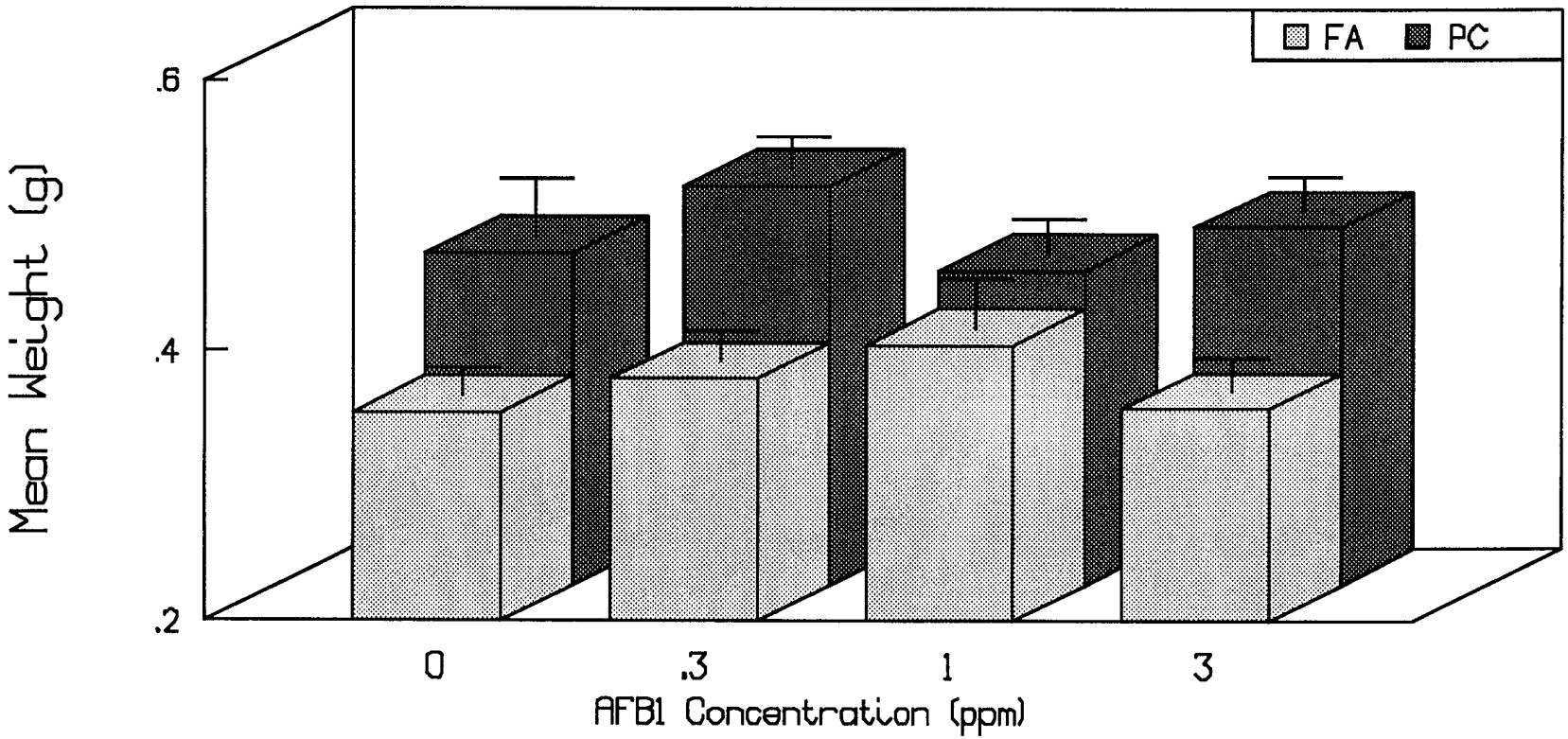
Table 3-6. Pilot Study Histopathologic Analysis of Control Medaka by Diet and Gender: Summary of Hepatic Foci of Cellular Alteration and Neoplasms

	EXPOSURE						TYPE								
	Diet	Conc.	N	Statistic	MBE	FCA	aph	bph	eph	epr	egr	fom	gly	vac	NEO
Female	FA	0	56	# examined	56	56									56
				# w/lesion	0	11	1	0	0	0	1	3	3	4	1
				freq	0	.1964	.02				.02	.05	.05	.07	.0179
Male	FA	0	49	# examined	42	42									42
				# w/lesion	1	11	0	0	0	1	5	3	3	3	1
				freq	.0238	.2619					.12	.07	.07	.07	.0238
Female	PC	0	25	# examined	25	25									25
				# w/lesion	1	4	0	0	0	0	0	0	0	4	1
				freq	.04	.16								.16	.04
Male	PC	0	18	# examined	15	15									15
				# w/lesion	0	1	0	0	0	0	0	0	0	1	0
				freq	0	.0667								.07	0

MBE = mixed basophilic and eosinophilic cells
FCA = foci of cellular alteration
aph = amphophilic
bph = basophilic
eph = eosinophilic
epr = eosinophilic, with large proteinaceous blebs
egr = eosinophilic, with small proteinaceous blebs
fom = foamy foci (probably small lipid droplets)
gly = glycogen-rich foci
vac = vacuolated foci (probably large lipid foci

LEGENDS FOR FIGURES

Figure 3-6. Histogram showing mean body weights of medaka fed either the F/A or PC-diets containing 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. Six month sampling from the AFB_1 pilot study. The data from which this histogram was constructed are found in Appendix 1.


FA
PC
.6
.4
.2
Mean Weight (g)
0
.3
1
3
AFB1 Concentration (ppm)

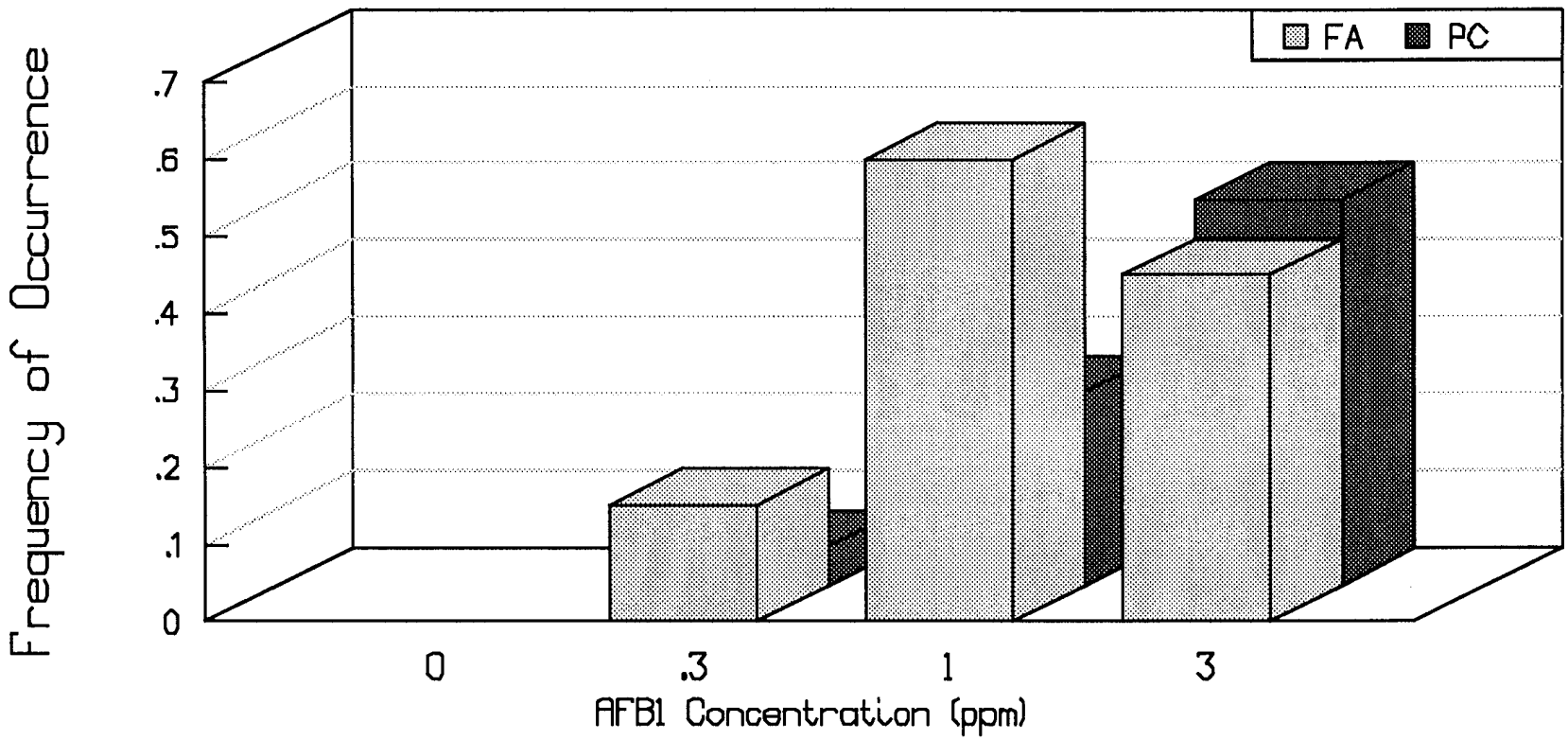
The frequency of occurrence for microscopic lesions of livers showing mixed basophilic and eosinophilic cells is shown (Fig. 3-7). This lesion, present at very low frequency in controls, was seen to increase in a concentration dependent fashion (note especially PC fed fish) with level of AFB_1 in the diet. This lesion, regarded as a reaction to toxicity, differed from the FCA in that the mixed lesion appears in mosaic fashion across the liver section. This lesion characterized the majority of medaka sampled during AFB_1 exposure.

Frequency of occurrence of FCA as a function of diet and AFB_1 concentration is shown in figure 3-8. For FA-fed fish, FCA frequency of occurrence for 0.3 ppm is nearly identical to that for PC fed fish at 1.0 ppm AFB_1. Promotional properties of F/A-diet are suggested by higher frequency in controls (0.0 ppm concentration FA versus PC) and at 0.3 ppm.

The frequency of occurrence of hepatic neoplasms as a function of diet and AFB_1 concentration is shown in figure 3-9. An apparent dose response relationship exists between frequency of neoplasms and increasing carcinogen concentration.

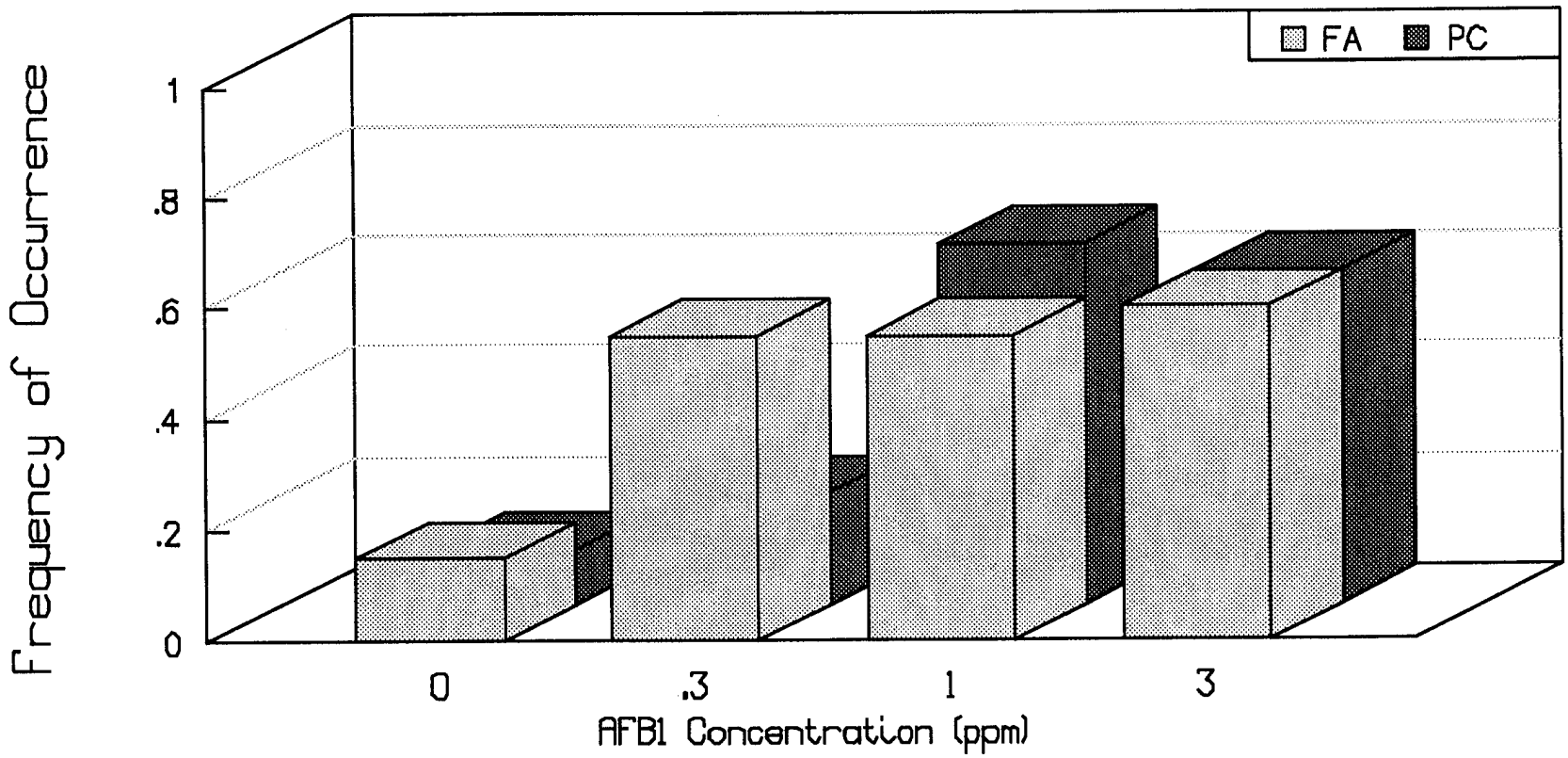
LEGENDS FOR FIGURES

Figure 3-7. Histogram of frequency of occurrence of mixed baso- and eosinophilic cells. Six month sampling, AFB_1 pilot study. The data from which this histogram was derived are found in Appendix 1. Medaka were fed either the F/A or PC-diets containing 0.3, 1.0 or 3.0 ppm of AFB_1.


FA
PC
Frequency of Occurrence
.7
.6
.5
.4
.3
.2
.1
0
0
.3
1
3
AFB1 Concentration (ppm)

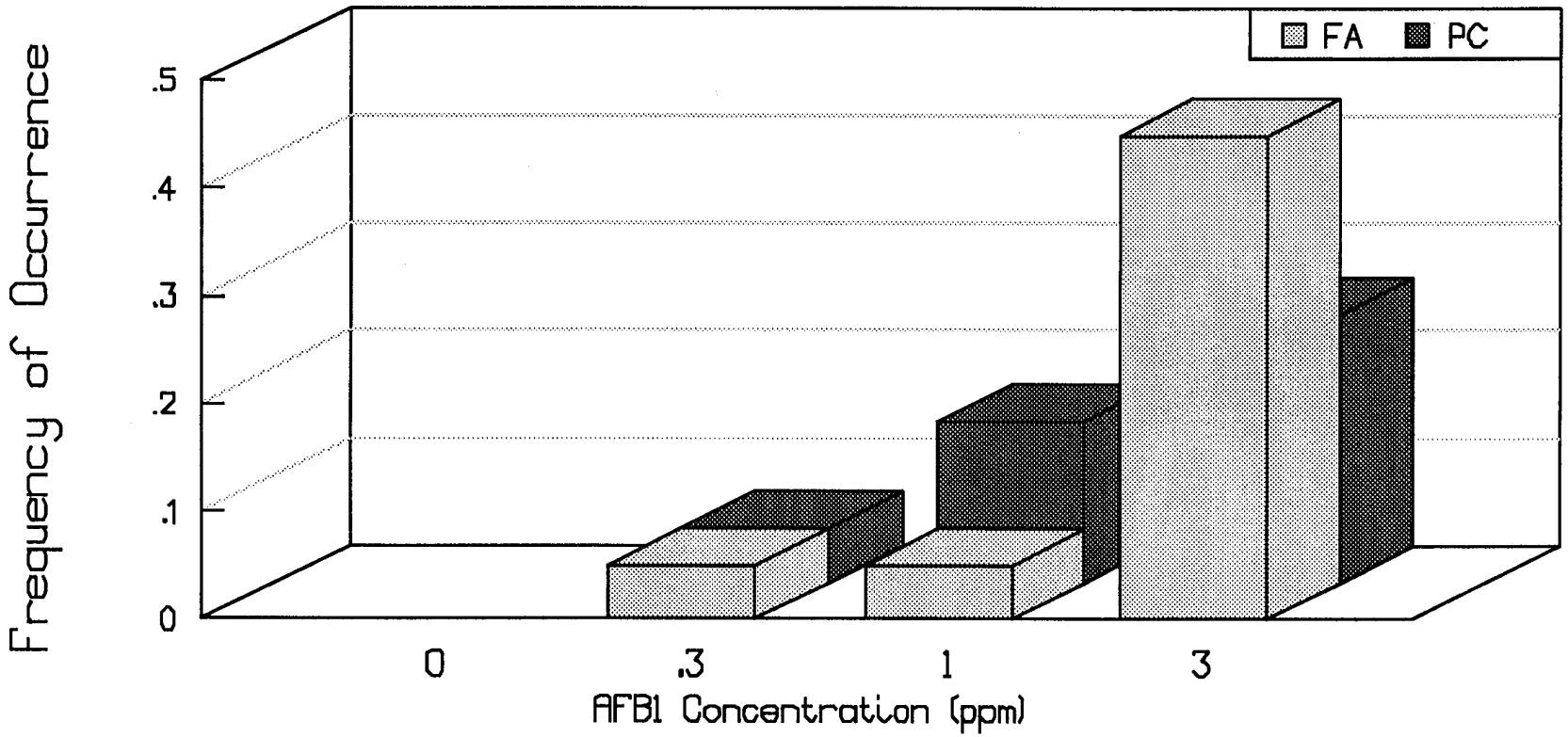
LEGENDS FOR FIGURES

Figure 3-8. Frequency of occurrence of foci of cellular alteration as a function of diet and AFB_1 concentration. Six month sampling of AFB_1 pilot study. Medaka were fed either F/A or PC-diet and exposed to 0.0, 0.3, 1.0 or 3.0 ppm of AFB_1.


FA
PC
Frequency of Occurrence
1
.8
.6
.4
.2
0
0
.3
1
3
AFB1 Concentration (ppm)

LEGENDS FOR FIGURES

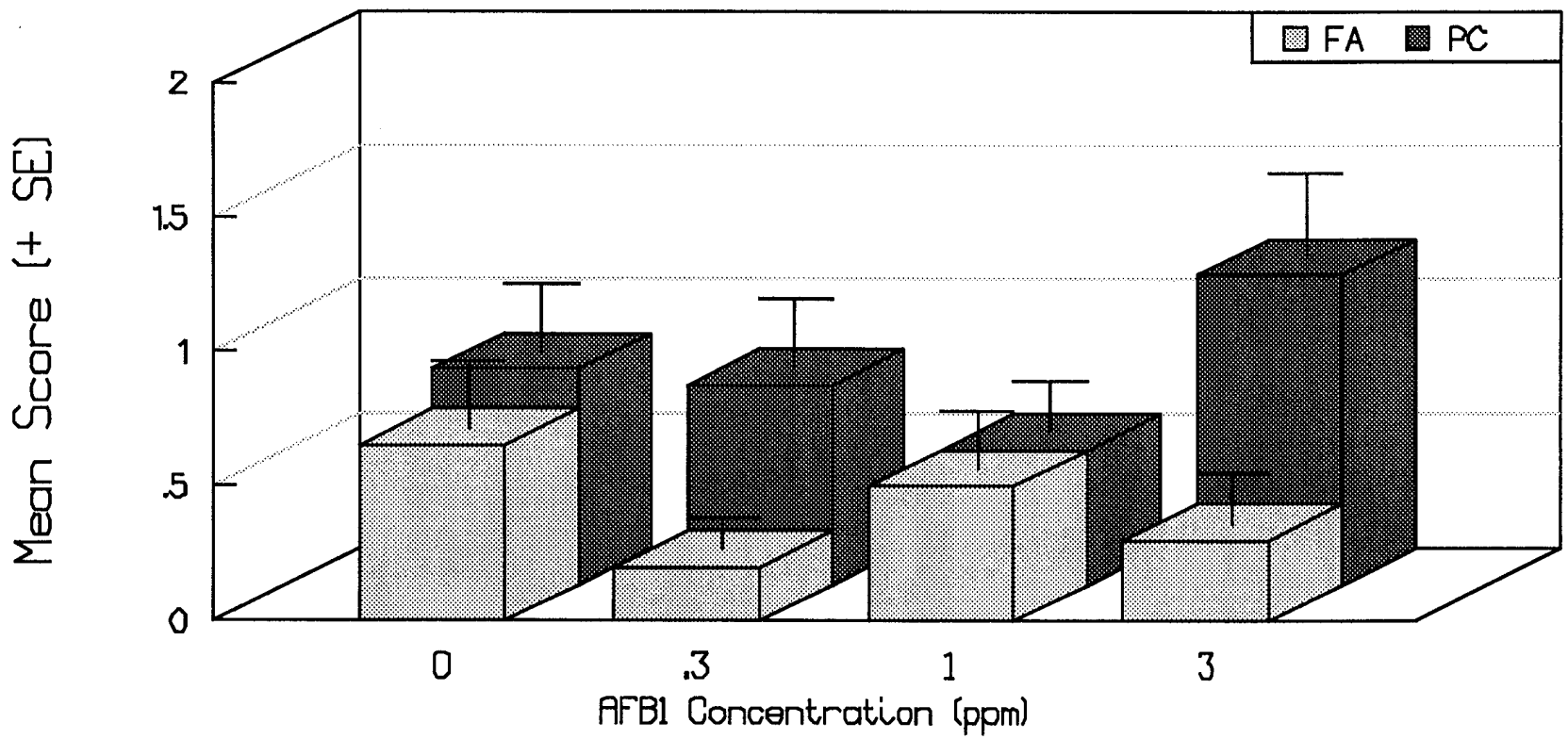
Figure 3-9. Frequency of occurrence of hepatic neoplasms in medaka fed F/A or PC-diet and exposed to 0.0, 0.3, 1.0, or 3.0 ppm dietary AFB_1 for six months. Six month sampling of AFB_1 pilot study. The actual data from which the histogram was constructed are found in Appendix 1.


FA
PC
Frequency of Occurrence
.5
.4
.3
.2
.1
0
0
.3
1
3
AFB1 Concentration (ppm)

Other lesions were read and frequencies recorded in medaka of both diets exposed to carcinogen. Mean severity scores for disseminated mycobacteriosis (DM) are presented in histogram form in figure 3-10. For FA medaka, highest mean severity score occurred in controls. PC-fed medaka differed in their scores with control scores being as high or higher than those of the lower AFB_1 groups but less than the mean score for the 3.0 ppm group. This suggests that factors other than AFB_1 working to influence DM scores. Mean severity scores for spongiosis hepatis are shown in figure 3-11. Apparently frequency of lesion was related in dose response association with carcinogen concentration in diet. However, response was greater in control and in two out of three treatment levels in FA versus PC fish. Atrial phagocyte hypertrophy (APH) as mean severity scores is shown in figure 3-12. For the FA fed fish, slight increase in severity occurred with increasing level of dietary AFB_1. For PC fed fish, the score for control fish was as high as that for all treatment groups. Factors other than, or in addition to AFB_1 seem to be influencing this lesion in PC fed fish. The pattern for PC fed fish was similar to that for DH. The mean severity scores for hepatocellular glycogen (HG) are shown in figure 3-13. Scores were lower in controls of FA fish than in their exposure groups. This indicates factors other than AFB_1 may influence this parameter in a negative fashion. The stairstep response with increasing AFB_1 indicates that the carcinogen concentration inversely affects this parameter. The PC fed fish show scores for controls that are higher than two of the three carcinogen groups indicating a similar inverse relationship.

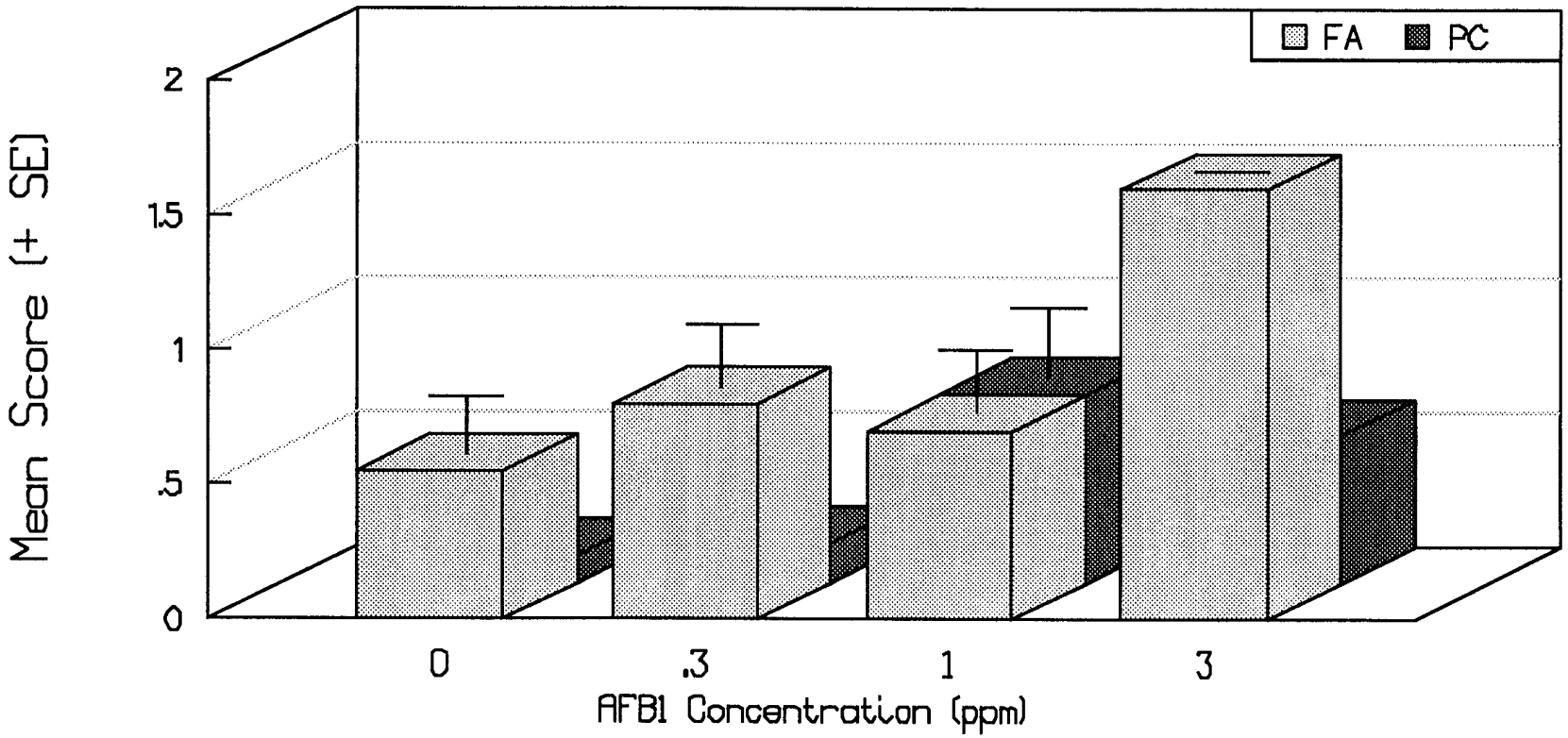
LEGENDS FOR FIGURES

Figure 3-10. Mean severity score for mycobacteriosis in medaka fed F/A or PC-diet and exposed to 0.0, 0.3, 1.0 or 3.0 ppm dietary AFB_1 for six months. Six months sampling; pilot AFB_1. The actual data from which the histogram was constructed are found in Appendix 1.


FA
PC
Mean Score (+ SE)
2
1.5
1
.5
0
0
.3
1
3
AFB1 Concentration (ppm)

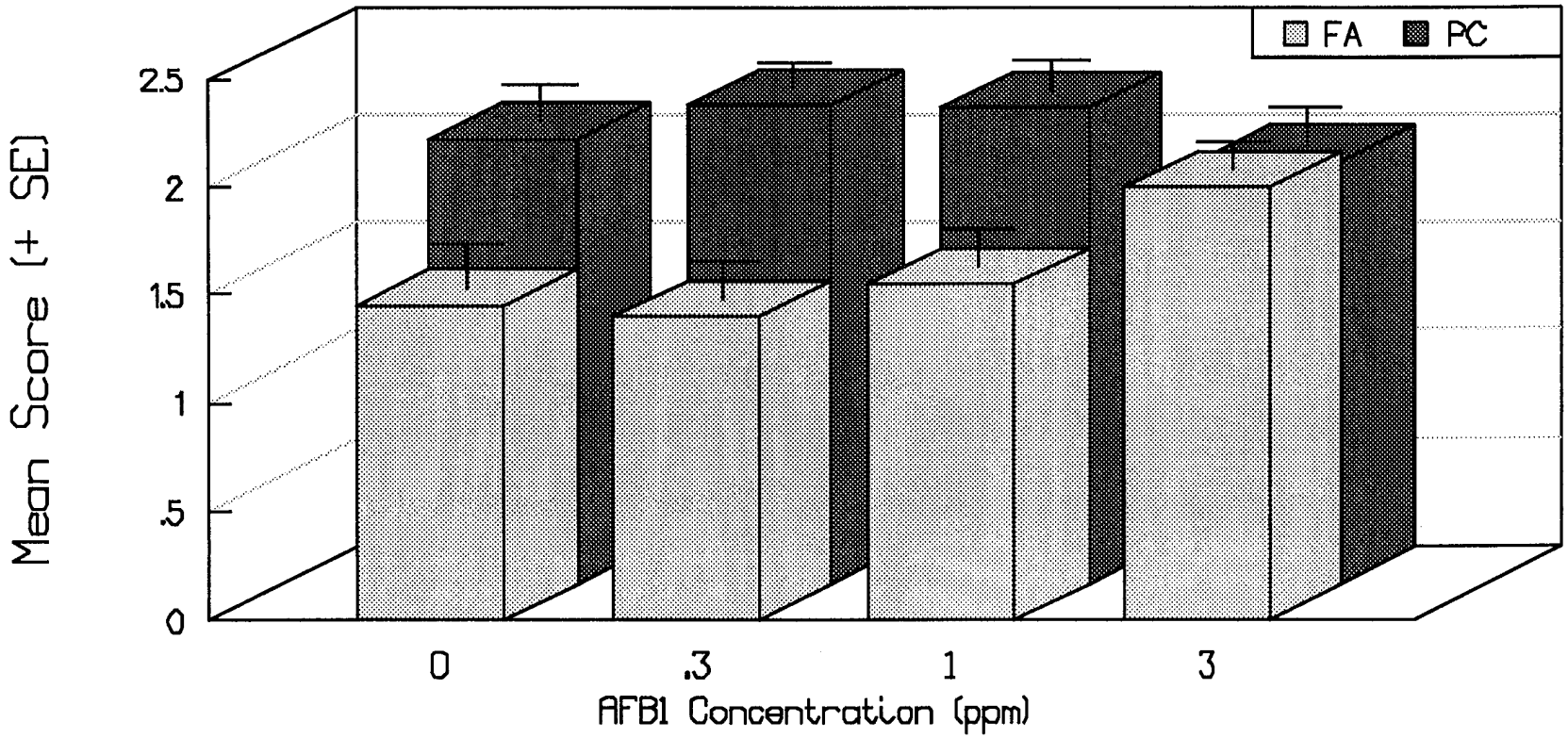
LEGENDS FOR FIGURES

Figure 3-11. Mean severity score for spongiosis hepatis in medaka fed F/A or PC-diet and exposed to 0.0, 0.3, 1.0 or 3.0 ppm dietary AFB_1 for six months. Six months sampling; pilot AFB_1. The actual data from which the histogram was constructed are found in Appendix 1.


FA
PC
Mean Score (+ SE)
2
1.5
1
.5
0
0
.3
1
3
AFB1 Concentration (ppm)

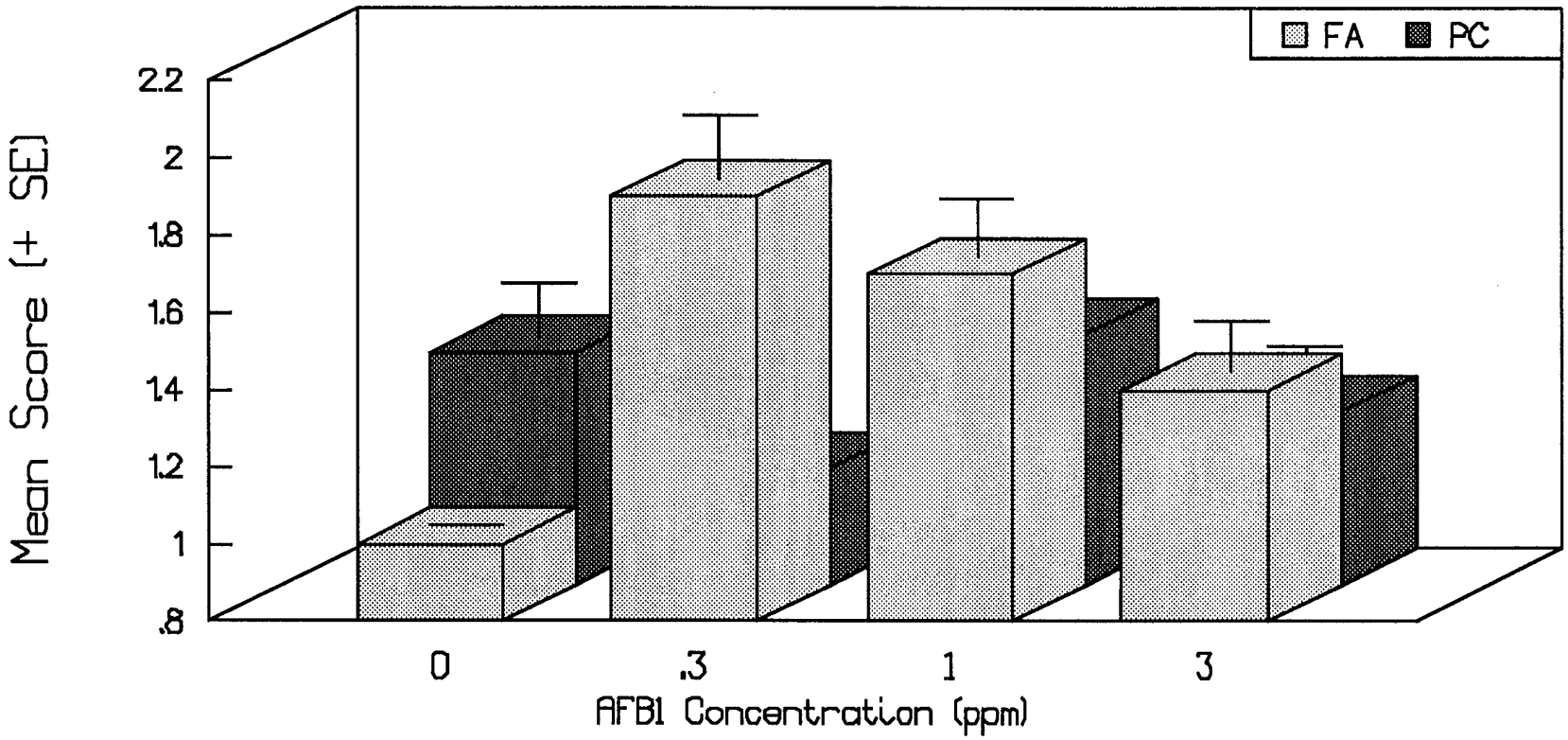
LEGENDS FOR FIGURES

Figure 3-12. Mean severity score for atrial phagocyte hypertrophy. Fish were fed either the F/A or PC-diets containing 0.0, 0.3, 1.0 or 3.0 ppm AFB_1 for six months. Six months sampling; pilot AFB_1. Actual data from which the histogram was constructed are found in Appendix 1.


FA
PC
Mean Score (+ SE)
2.5
2
1.5
1
.5
0
0
.3
1
3
AFB1 Concentration (ppm)

LEGENDS FOR FIGURES

Figure 3-13. Hepatocellular glycogen - mean score. Fish were fed the F/A or PC-diets for six months. They were exposed to either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. Six months sampling; pilot AFB_1. Actual data from which the histogram was constructed are found in Appendix 1.

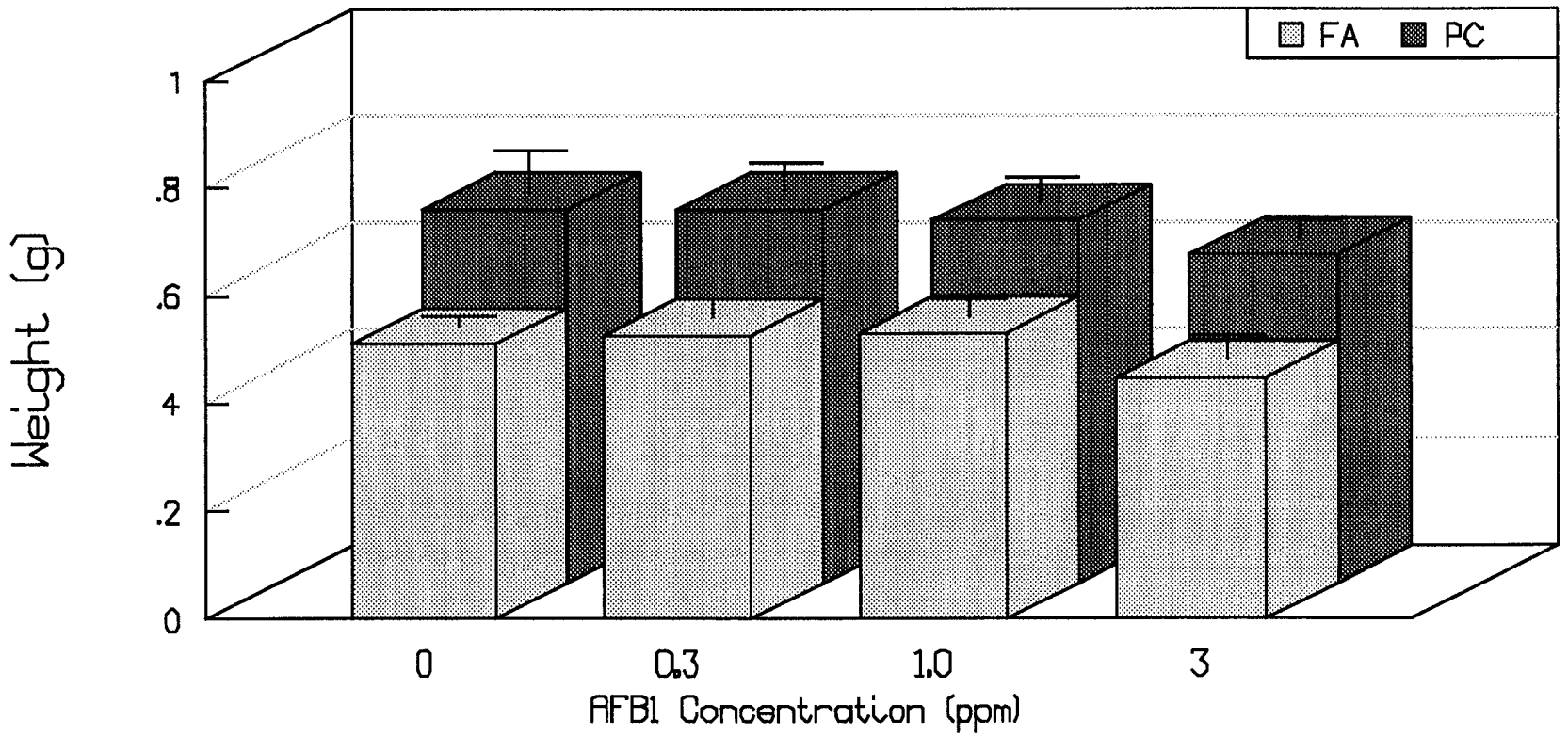

FA
PC
Mean Score (+ SE)
2.2
2
1.8
1.6
1.4
1.2
1
.8
0
.3
1
3
AFB1 Concentration (ppm)

Figures 3-14 through 3-20 present results of the histopathologic analysis of medaka sampled at nine months in the AFB_1 pilot study. These fish have had six months of dietary exposure to the carcinogen then, three months of recovery (growout) under standard conditions with no additional carcinogen exposure. Weights are averaged for medaka of each dietary and carcinogen concentration group and are presented as a histogram in figure 3-14. Note that the weights of medaka on the PC-diet exceed those of each of their FA counterparts. A slight decreasing trend with each diet and increasing AFB_1 concentration is shown. FCA frequency of occurrence is presented as a histogram in figure 3-15. Apparent differences are seen in FA versus PC fed medaka. First, for the FCA of FA-fed fish, foci are at higher frequency in fish given the lower AFB_1 concentration, and, as one proceeds to the next higher and the highest, the frequency drops off. For the PC-fed fish, an opposite effect is seen. The increasing levels of AFB_1 are associated with increasing frequency of FCA. If some FCA of the FA-fed fish are promoted into tumors, then frequency of FCA would appear to diminish with increasing AFB_1 concentration. In the six months group, the frequency of FCA in PC-fed fish lagged behind that of the FA-fed fish. Perhaps a longer time would be necessary for the PC-fed group to show similar relationship to that of the FA-fed fish. Either way, a positive dose response with FCA is seen as AFB_1 concentration is increased. The histogram for hepatic neoplasm frequency is shown in figure 3-16. A stairstep relationship is shown for the FA-fed group; as AFB_1 increases, so does neoplasm frequency. Compare 3-16 to 3-15 and the patterns suggest that FCA frequency diminishes as tumor frequency increases. Interestingly, no tumors were seen in 1.0 ppm AFB_1, PC-fed medaka. A small number of survivors may account for some of this surprising response. If the other two concentrations are considered, increasing AFB_1 concentration also increases tumor frequency in

PC-fed and carcinogen exposed fish. Figure 3-17 presents a histogram for mean severity scores for DM in the nine months sampling of the pilot study. Control groups of both diets had higher severity scores than their corresponding treatment groups. For each diet, increasing the concentration of carcinogen diminished the DH severity score. Mean severity scores for SH are shown in figure 3-18. The pattern for increased SH severity score in medaka fed the F/A-diet is continued at this later sampling. Control FA-fed fish showed the lesion while control PC fish did not. PC-diet fed fish only showed SH if they were exposed to AFB_1. Both AFB_1 and some factor present in F/A-diet alone are associated with SH. APH mean severity score is shown in figure 3-19. PC-diet with or without AFB_1 was associated with APH. APH severity was higher in all PC groups versus their FA corresponding groups. Mean severity score for hepatocellular glycogen is shown in figure 3-20. Compared to the corresponding feature in the six months sampling, return of the fish to standard recirculating aquaria conditions has improved this parameter. Controls of both groups have greater areas of the cytoplasm occupied by glycogen now indicating that some stress has been removed. The dose response seen in the FA-fed groups at six months is no longer seen. This indicates that active exposure to AFB_1 was associated with glycogen depletion.

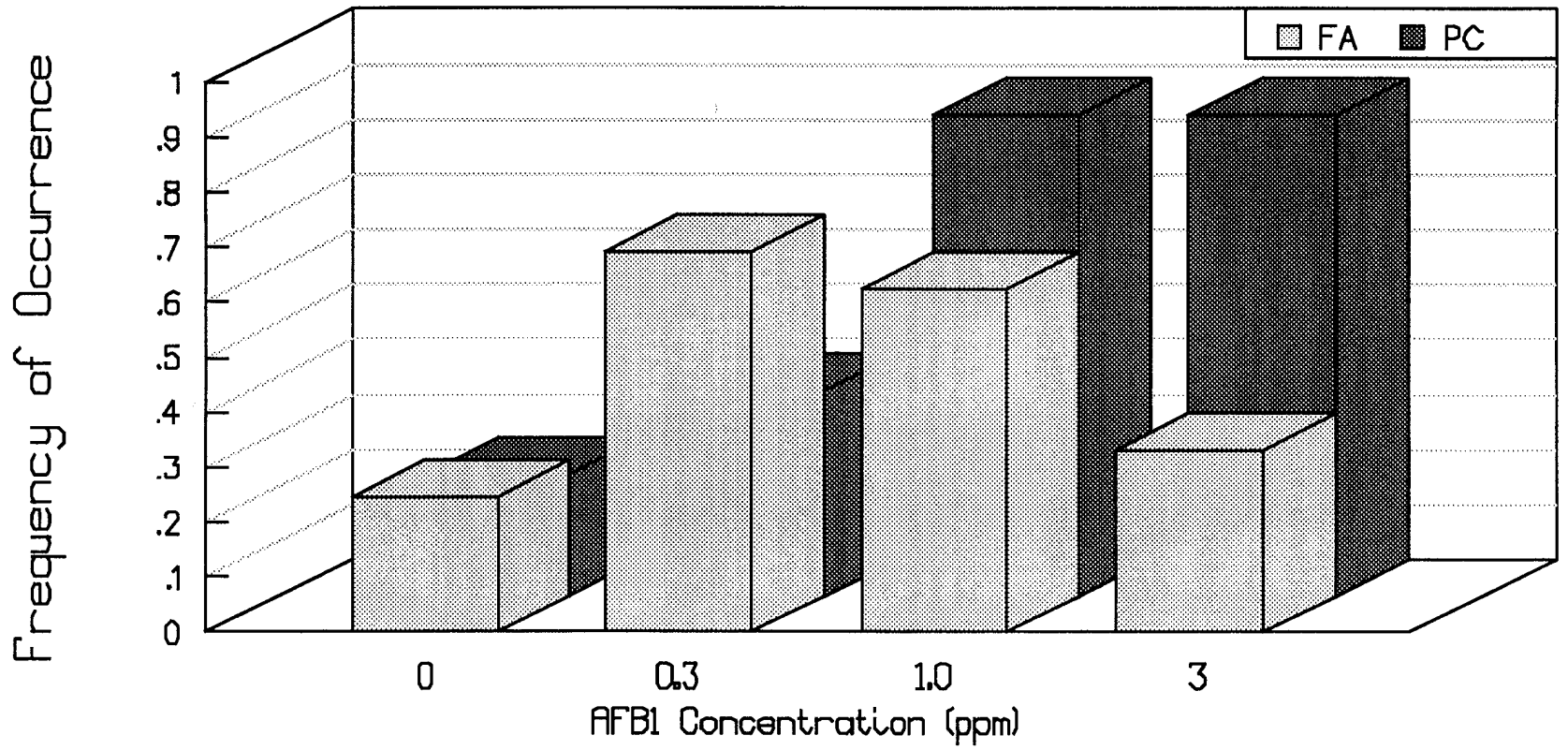
LEGENDS FOR FIGURES

Figure 3-14. Histogram of body weight of medaka by diet and by AFB_1 concentration. Fish were fed a diet containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1 for six months and then allowed to recover under standard aquarium conditions for an additional three months. Nine months sampling; pilot AFB_1. Actual data to construct this histogram are shown in Appendix 2.


FA
PC
Weight (g)
1
.8
.6
4
.2
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

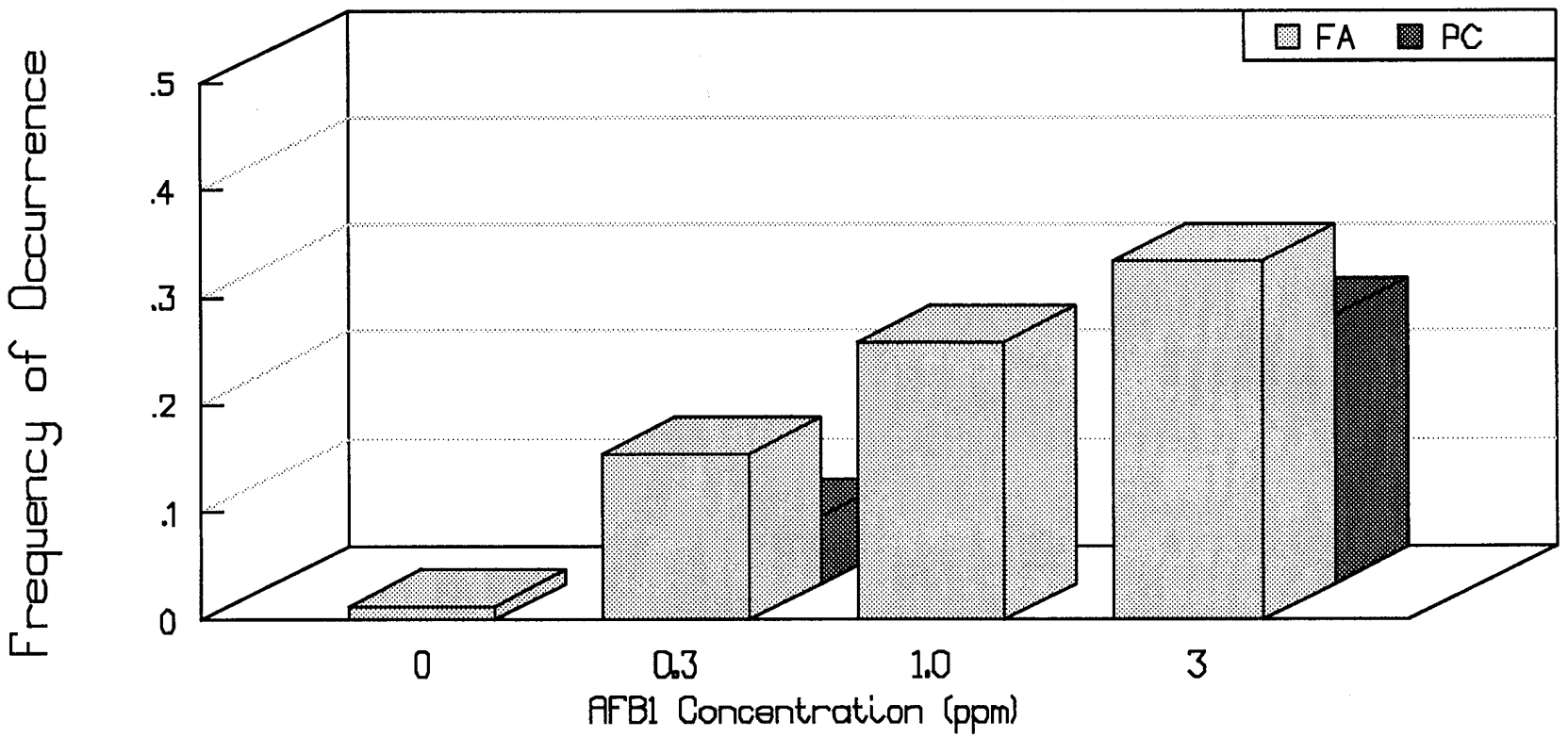
LEGENDS FOR FIGURES

Figure 3-15. Frequency of occurrence of foci of cellular alteration in livers of medaka. Medaka were fed either the F/A or PC-diet containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. Fish were fed the respective diets for six months and then allowed to recover under standard aquarium conditions for an additional three months. Nine months sampling; pilot AFB_1. Actual data used to construct this histogram are found Appendix 2.


FA
PC
Frequency of Occurrence
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

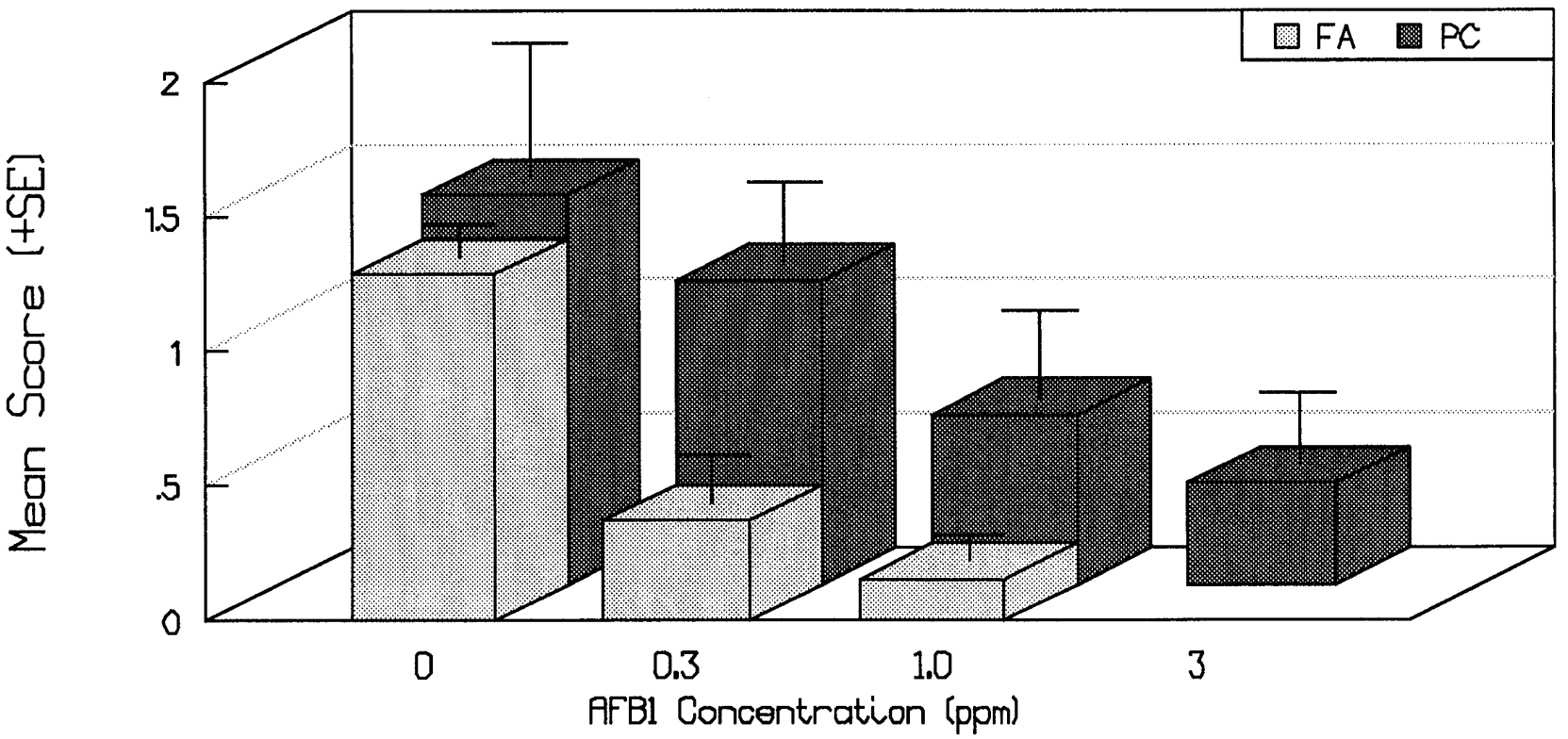
LEGENDS FOR FIGURES

Figure 3-16. Frequency of occurrence of hepatic neoplasms in medaka. Medaka were fed the F/A or PC-diets containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1 for six months and then allowed to recover under standard aquarium conditions for an additional three months. Nine months sampling; pilot AFB_1. The data actually used to construct this histogram are presented in Appendix 2.


FA
PC
Frequency of Occurrence
.5
.4
.3
.2
.1
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

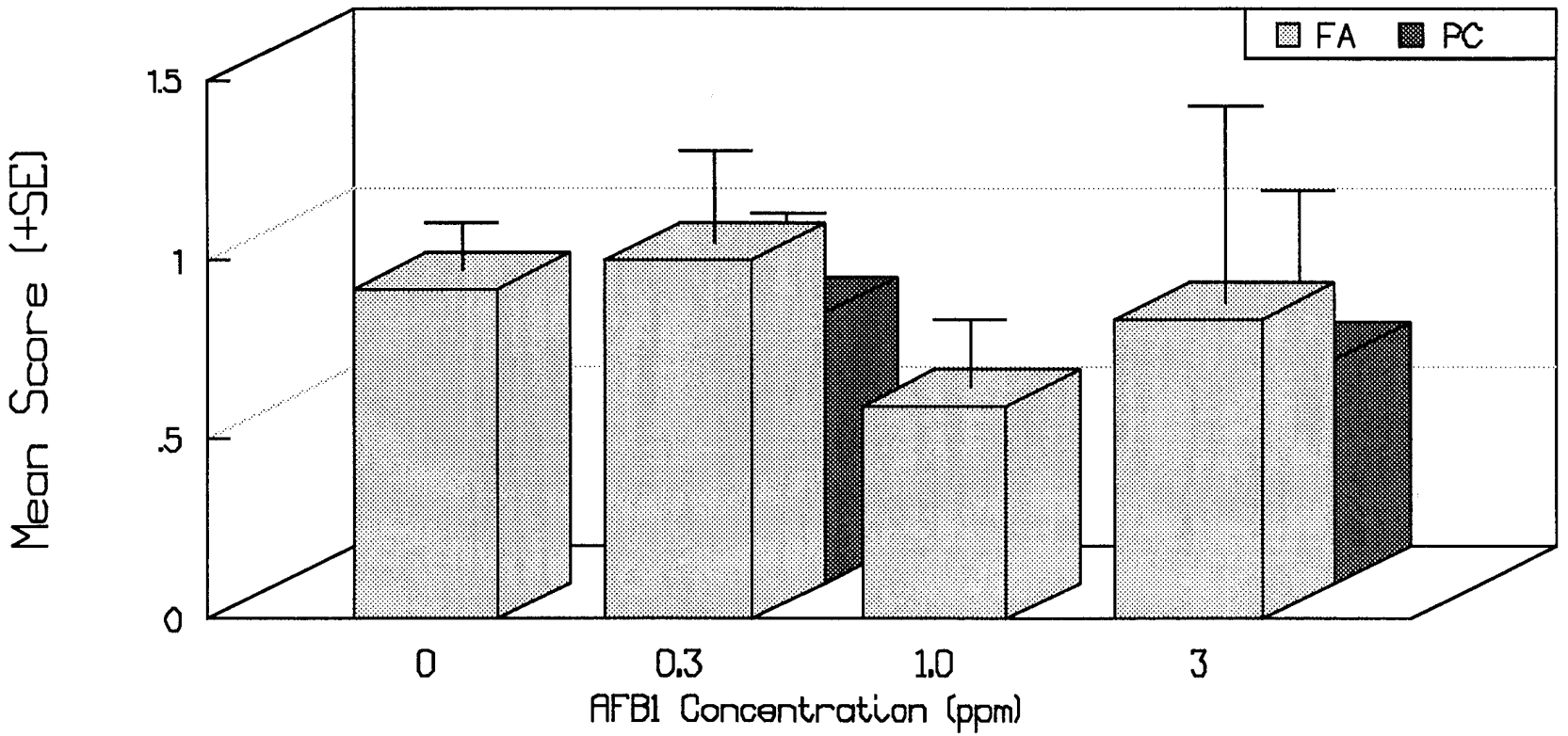
LEGENDS FOR FIGURES

Figure 3-17. Mean severity score for disseminated mycobacteriosis. Medaka were fed either the F/A or PC-diets containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. After exposure for six months, fish were placed in growout aquaria under standard culture conditions and allowed to recover for an additional three months prior to histologic sampling. Nine months sampling; pilot AFB_1. The numerical data used to construct the histogram is contained in Appendix 2.


FA
PC
Mean Score (+SE)
2
1.5
1
.5
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

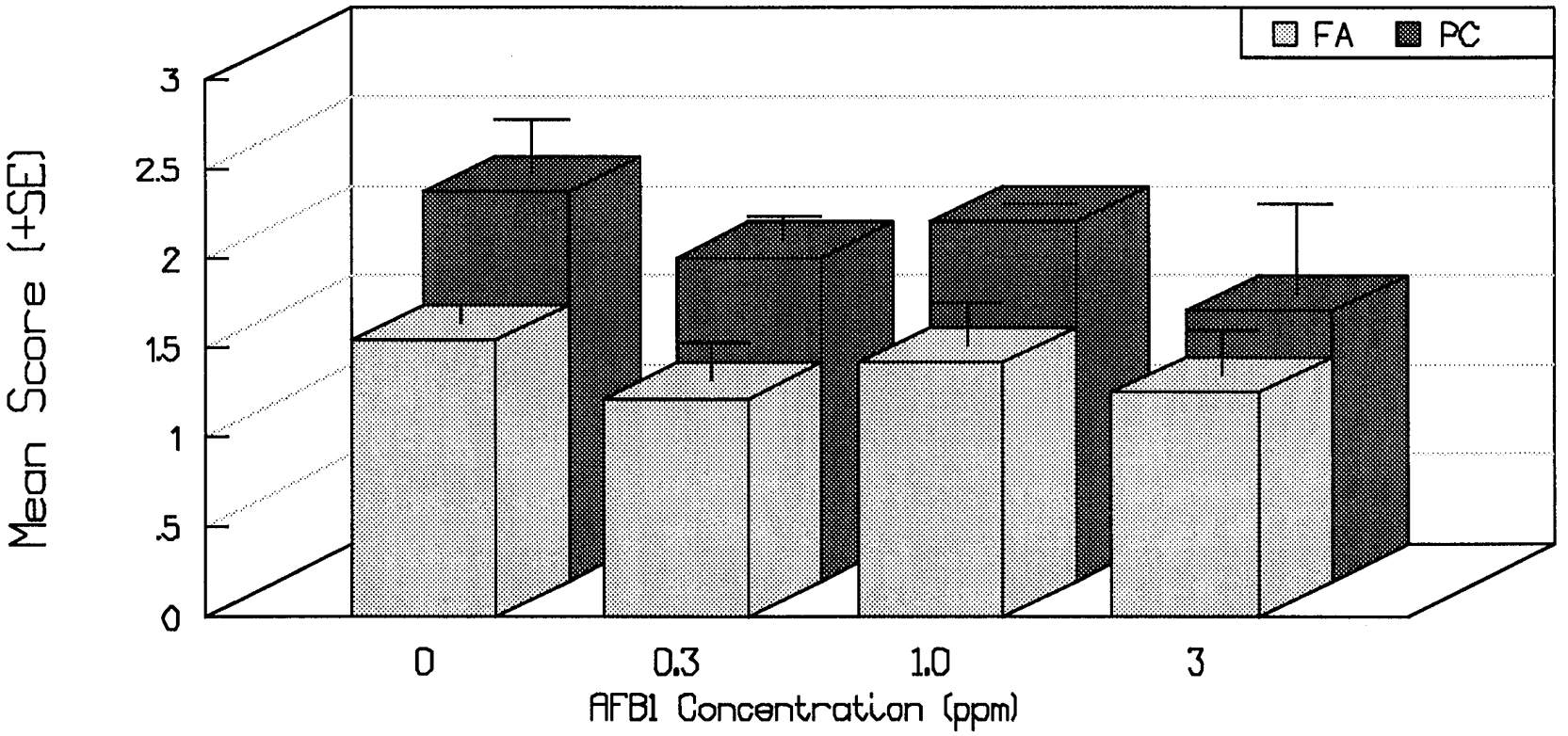
LEGENDS FOR FIGURES

Figure 3-18. Mean severity score for spongiosis hepatis. Medaka were fed either the F/A or PC-diets containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. Fish were exposed to the carcinogen for six months and then placed in clean water for recovery for an additional three months. Nine months sampling; pilot AFB_1. The actual data used to construct the histograms is contained in Appendix 2.


FA
PC
1.5
1
.5
0
Mean Score (+SE)
0
0.3
1.0
3
AFB1 Concentration (ppm)

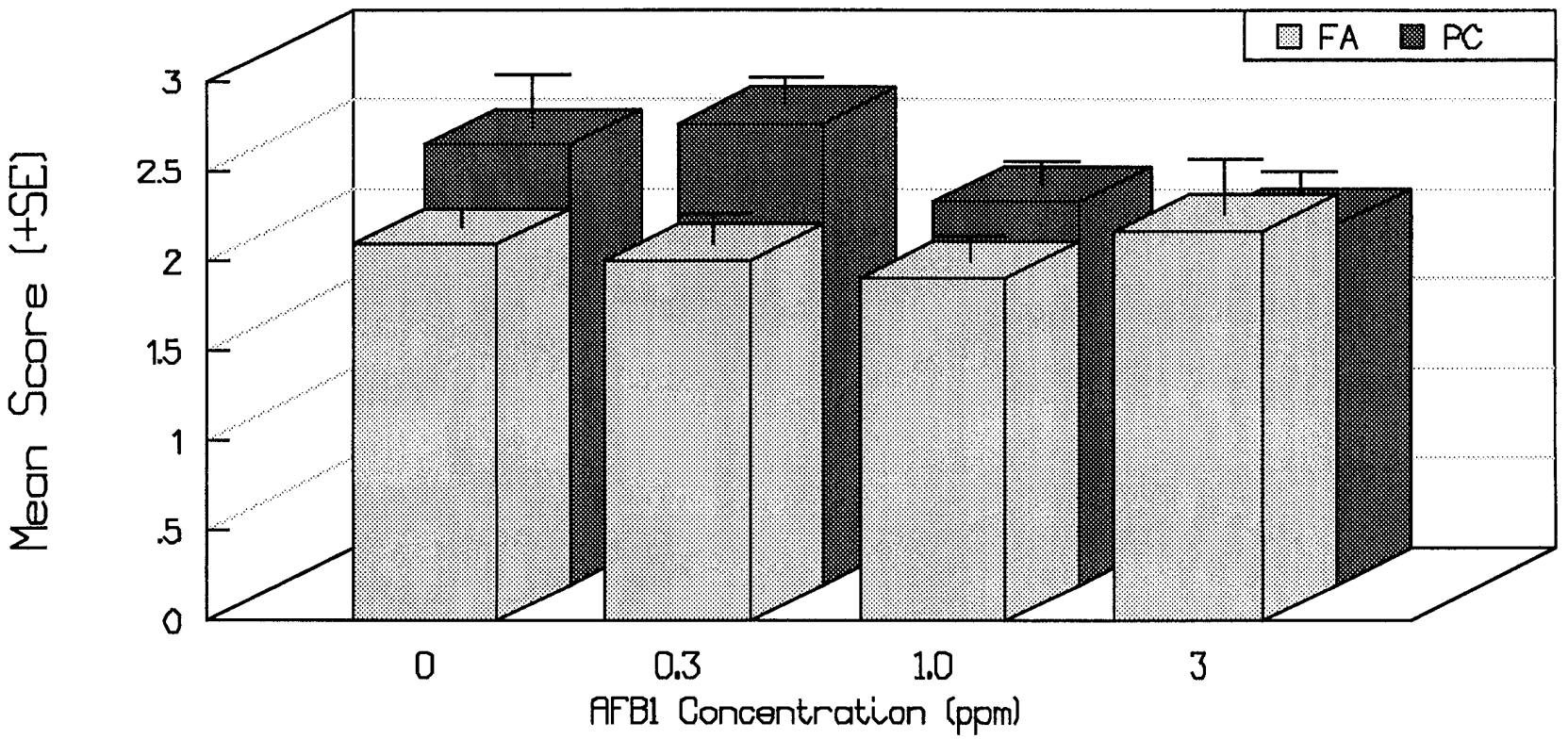
LEGENDS FOR FIGURES

Figure 3-19. Mean severity score for atrial phagocyte hypertrophy. Medaka were fed either the F/A or PC-diets containing either 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. Duration of exposure was six months followed by three months of growout under standard laboratory conditions. Nine months sampling; pilot AFB_1. Actual data used to construct the histograms are contained in Appendix 2.


FA
PC
Mean Score (+SE)
3
2.5
2
1.5
1
.5
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

LEGENDS FOR FIGURES

Figure 3-20. Mean severity score for hepatic glycogen. Nine months sampling; pilot AFB_1. Medaka were fed either the F/A or PC-diets. They were exposed for six months through the dietary route to 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. After the exposure, fish were placed in standard aquaria and allowed to growout for a period of an additional three months. Actual data used to construct the histogram shown are in Appendix 2.


FA
PC
Mean Score (+SE)
3
2.5
2
1.5
1
.5
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

Results illustrated in text figures 3-20 - 3-26 are assembled from all medaka of the pilot study including, six and nine month samplings and all of the fish which died during the test. Figure 3-21 presents findings for frequency of occurrence of FCA. A dose-dependent effect is shown by fish fed FA and by those fed PC-diets. However, in medaka fed the F/A-diet, FCA frequency of occurrence appears to decrease with exposure to the highest AFB_1 concentration. FCA frequency of occurrence increased with increasing AFB_1 concentration.

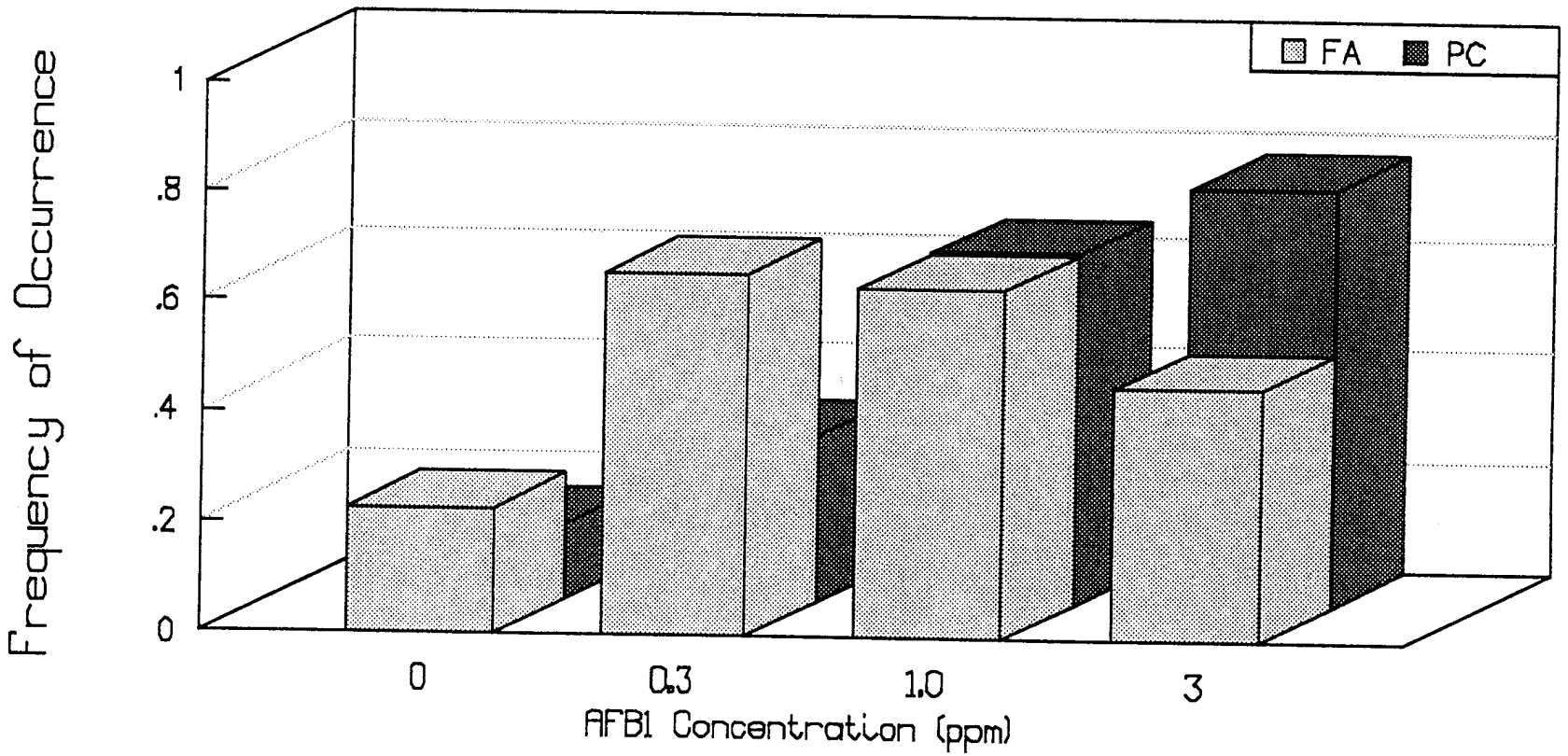
Figure 3-22 presents neoplasm positive medaka fed F/A-diet. Six and nine month samplings are included along with mortalities occurring during the test. A single hepatic adenoma was seen in one control fish. Greatest number of tumor bearing fish was in the 3.0 ppm group. Note that fish dying during the test accounted for the most tumor bearing fish in the 0.3 and the 3.0 ppm groups. The large number of tumor bearing fish in the 3.0 ppm group may indicate that FCA (see fig. 3-20) had been promoted to tumors and could explain the decreasing incidence of FCA seen after nine months. When frequency of tumor bearing fish from the pilot study was determined for those fish fed the PC-diet, a dose response was seen (fig. 3-23). Also, fish dying during the test were the major group of tumor bearing fish. Figure 3-24 presents the number of tumor bearing fish as a function of diet and carcinogen concentration. The greatest frequency of tumor bearing fish was in the FA-3.00 AFB_1 group. Approximately the same frequency (20%) was seen for the two lower carcinogen levels in fish fed the F/A-diet. In those fed the PC-diet, a dose positive dose response was seen but at lower frequency (fig. 3-24).

Number of FA-fed fish dying during the test is shown in fig 3-25. The number dead by 180 days was distributed along AFB_1 concentration with $0.0 < 0.3 < 1.0 < 3.00$. This suggests that AFB_1 - associated toxicity was the major cause for deaths in the FA-fed fish. When an identical

treatment of the mortality is provided for fish fed the PC-diet (fig. 3-26), mortalities were not distributed along AFB_1 concentration. Rather, mortalities increased by this sequence, $0.3 < 0.0 \leq 3.0 < 1.0$. This suggests that AFB_1 concentration was not the only factor underlying mortality.

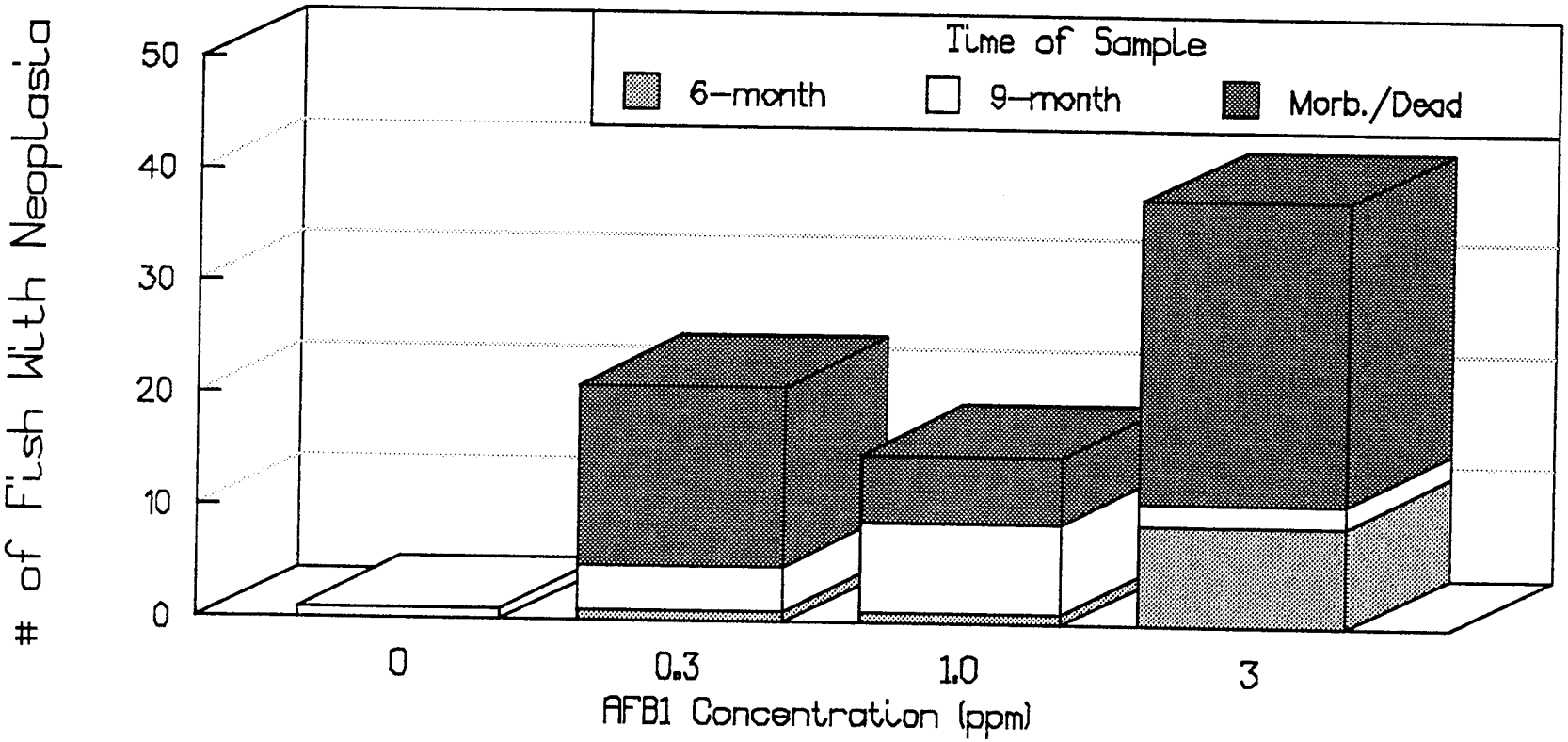
LEGENDS FOR FIGURES

Figure 3-21. Frequency of occurrence of foci cellular alteration in all medaka included in the pilot study. Nine months sampling; pilot AFB_1. Analysis included those fish that died during the study as well as those that were sampled at 6 and at 9 months. Fish were fed either the F/A or PC-diets containing 0.0, 0.3, 1.0, or 3.0 ppm AFB_1. The actual data from which the histogram was constructed are found in Appendix 2.


FA
PC
Frequency of Occurrence
1
.8
.6
.4
.2
0
0
0.3
1.0
3
AFB1 Concentration (ppm)

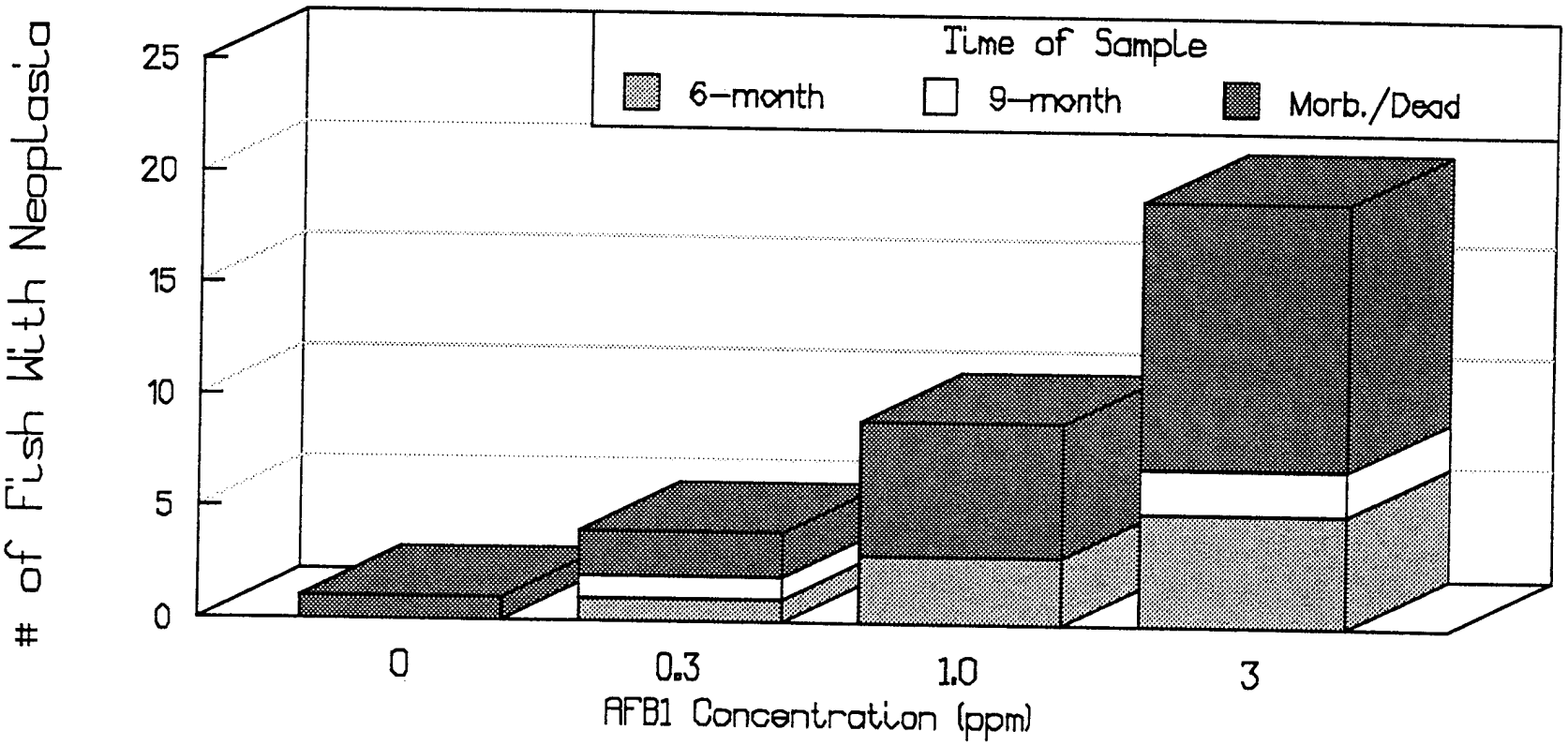
LEGENDS FOR FIGURES

Figure 3-22. Number of fish fed the F/A diet containing hepatic neoplasms. This histogram reveals fish from the 6-month, 9-month, and those that died during the test. Actual data used to construct the histogram are included in Appendix 2.


# of Fish With Neoplasia
50
40
30
20
10
0
Time of Sample
6-month
9-month
Morb./Dead
0
0.3
1.0
3
AFB1 Concentration (ppm)

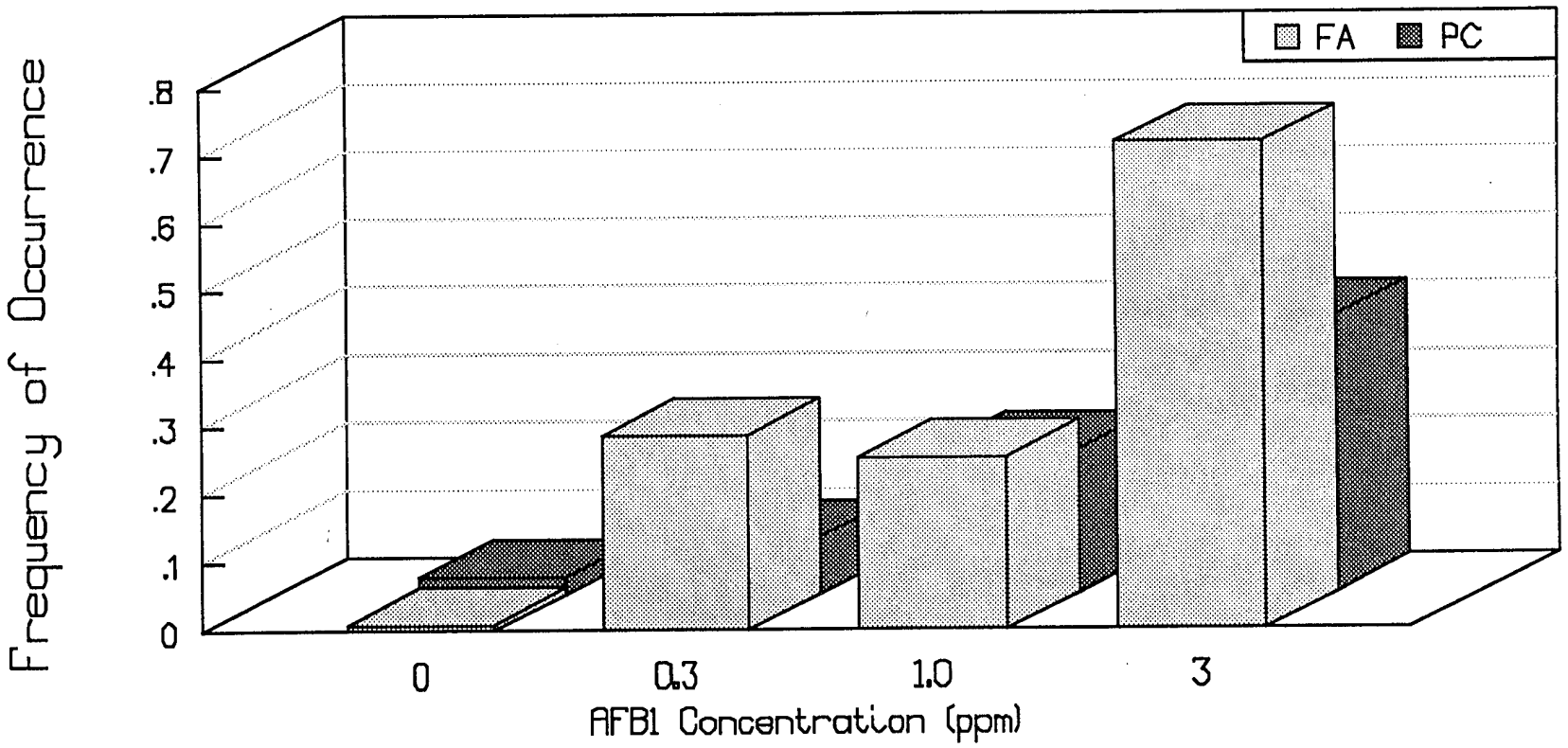
LEGENDS FOR FIGURES

Figure 3-23. Number of fish fed the PC-diet and containing hepatic neoplasms. Fish from both sampling periods as well as those that died during the test are included. Fish were exposed to AFB_1 for a period of six months and some of these were extended in under growout conditions for an additional period of three months. Exposure to AFB_1 was at 0.0, 0.3, 1.0, or 3.0 ppm. Actual data used to construct this histogram are shown in Appendix 2.


# of Fish With Neoplasia
25
20
15
10
5
0
Time of Sample
6-month
9-month
Morb./Dead
0
0.3
1.0
3
AFB1 Concentration (ppm)

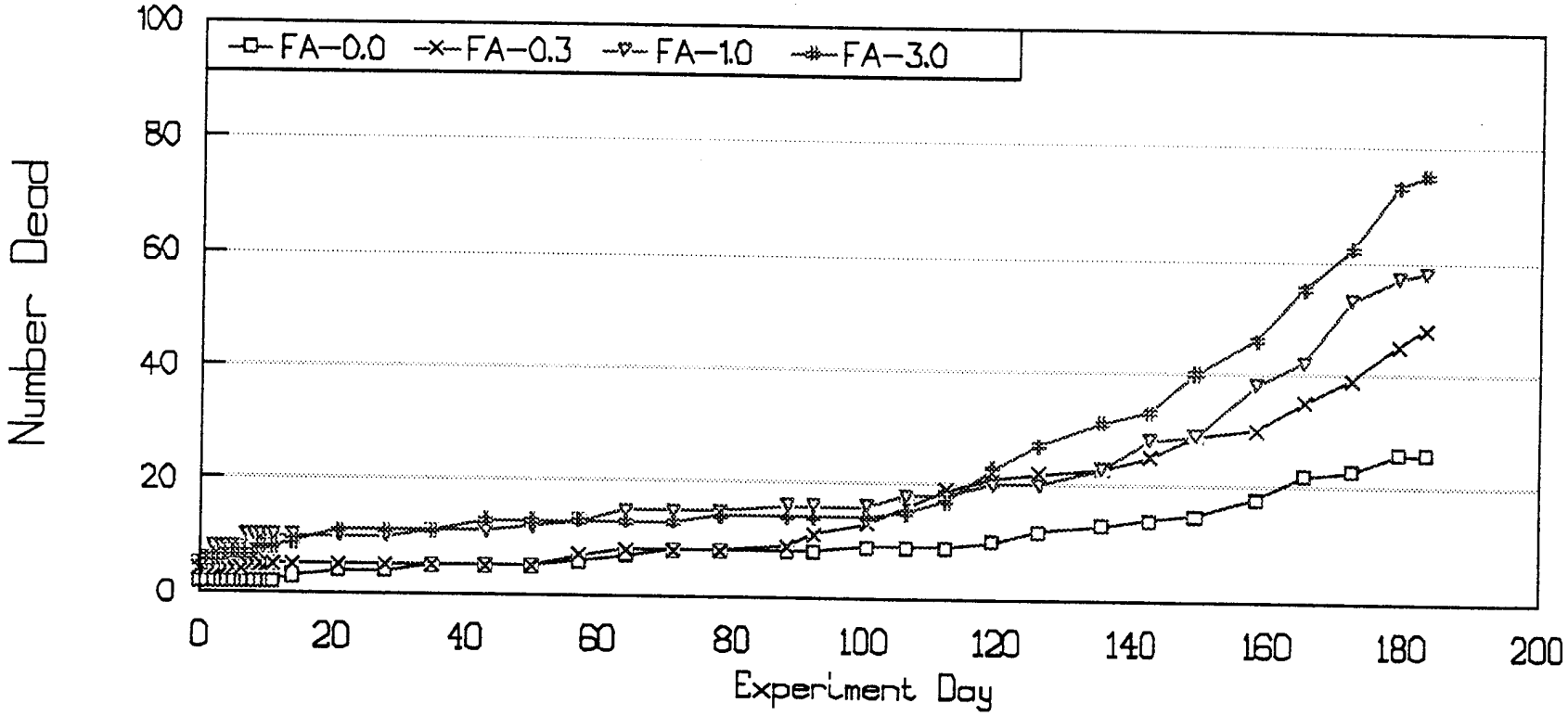
LEGENDS FOR FIGURES

Figure 3-24. Frequency of occurrence of neoplasms in fish fed either the F/A or PC-diets. This histogram compares effects of hepatic neoplasia as a function of diet. Fish were exposed to the carcinogen for six months and then some of them were allowed to recover for an additional three months. Also included are data from fish dying within the test. AFB_1 concentrations in the diet were 0.0, 0.3, 1.0, or 3.0 ppm.


FA
PC
Frequency of Occurrence
0
.1
.2
.3
.4
.5
.6
.7
.8
0
0.3
1.0
3
AFB1 Concentration (ppm)

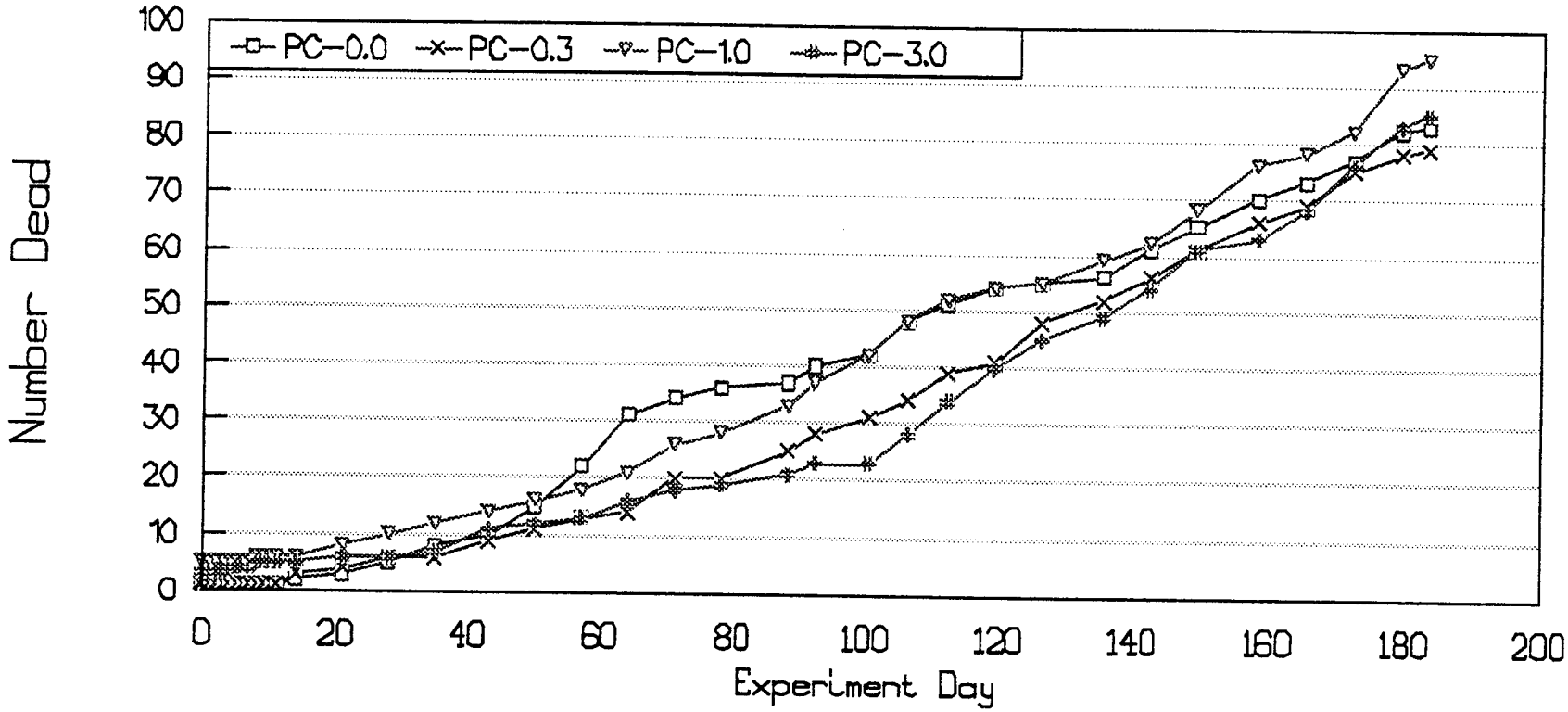
LEGENDS FOR FIGURES

Figure 3-25. Number of mortalities as a function of time in fish fed the F/A diet. Fish were fed 0.0, 0.3, 1.0, or 3.0 ppm of AFB_1. The mortalities include fish that died prior to the first sampling, fish from the first sampling, fish dying between the first and second samplings, fish from the second sampling, and those dying between the second sampling and the termination of the test. Actual data on mortalities are contained in Appendix 2.


FA–0.0
FA–0.3
FA–1.0
FA–3.0
Number Dead
0
20
40
60
80
100
0
20
40
60
80
100
120
140
160
180
200
Experiment Day

LEGENDS FOR FIGURES

Figure 3-26. The number of mortalities as a function of time during the test for fish fed the PC-diet. Fish were exposed to 0.0, 0.3, 1.0 or 3.0 ppm AFB_1. Mortality includes those fish dying between the start of the test and the first sampling, at the first sampling, between the first and second samplings, at the second sampling, and after this sampling and before the termination of the test. The actual numbers for cumulative mortality may be found within Appendix 2.


PC-0.0
PC-0.3
PC-1.0
PC-3.0
Number Dead
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120
140
160
180
200
Experiment Day

Micrographs Illustrating Histopathologic Alterations - Pilot Study - Representative alterations are figured in Plates 1-3. Other alterations such as FCA, hepatic neoplasms and other AFB_1 - associated lesions in extrahepatic sites are illustrated and described in Chapter 2 and in the definitive AFB_1 study described below. Plate 1, figure A shows appearance of head kidney from a control medaka fed the PC-diet. In this approximately one year old fish, multiple granulomata are seen in intertubular region. Inside lumina of renal tubules, we encountered macrophages and sloughed tubular epithelial cells. This kidney was from an animal with disseminated mycobacteriosis. Selective electron microscopy was performed on skeletal muscle of body wall in medaka with disseminated mycobacteriosis. An example of muscular involvement is shown (Plate 1, figure B). Bacteria have colonized the myocyte in the center of the field and are freely dispersed adjacent to mitochondria and vesicles.

Panhepatic toxicity was evidenced by formation of a mosaic of alternating basophilic and eosinophilic regions occurring across the liver section (Plate 2, figure A). This condition is different from FCA in that alterations occur in regularly repeating pattern and involve most or all of the section. FCA persisted after cessation of dietary AFB_1 exposure while panhepatic toxicity patterns did not. Since dietary AFB_1 exposure was continued for six months, it is likely that "hits" would continue to occur even within established lesions.

Plate 2, Figure B illustrates a mixed focus which we feel is due to the ongoing exposure overlaid on preexisting alterations. A focus with amphophilic, eosinophilic and basophilic phenotypes is shown (Plate 2, Figure B). These were not encountered in DEN-exposed fish (Chapter 2) where exposure to initiating carcinogen was brief and administered for a single, 48 hour duration.

An eosinophilic adenoma of the liver is shown in Plate 3, figure A. This well circumscribed mass contrasted with the basophilic focus seen in another area of the same liver section. Multiple hits were common in medaka chronically exposed to AFB_1.

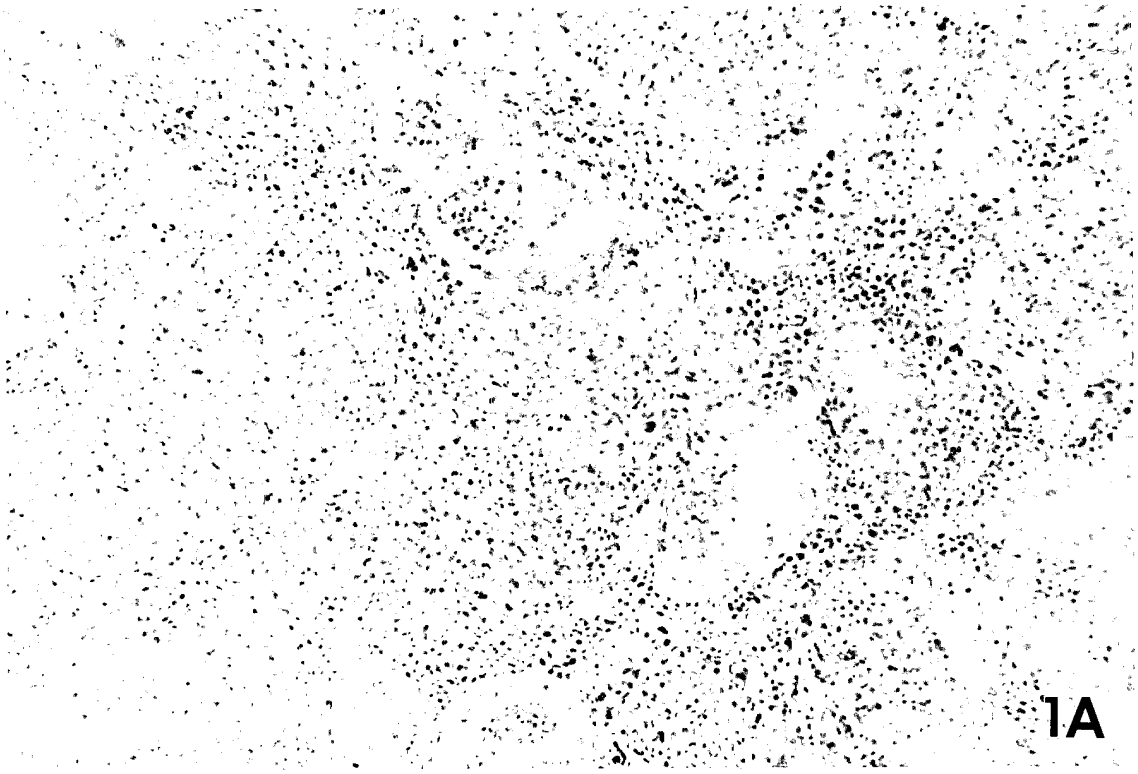
An adenoma of the gas gland of medaka is shown in Plate 3, figure B. This solid mass fills the cross sectional profile of that normally gas-filled structure.

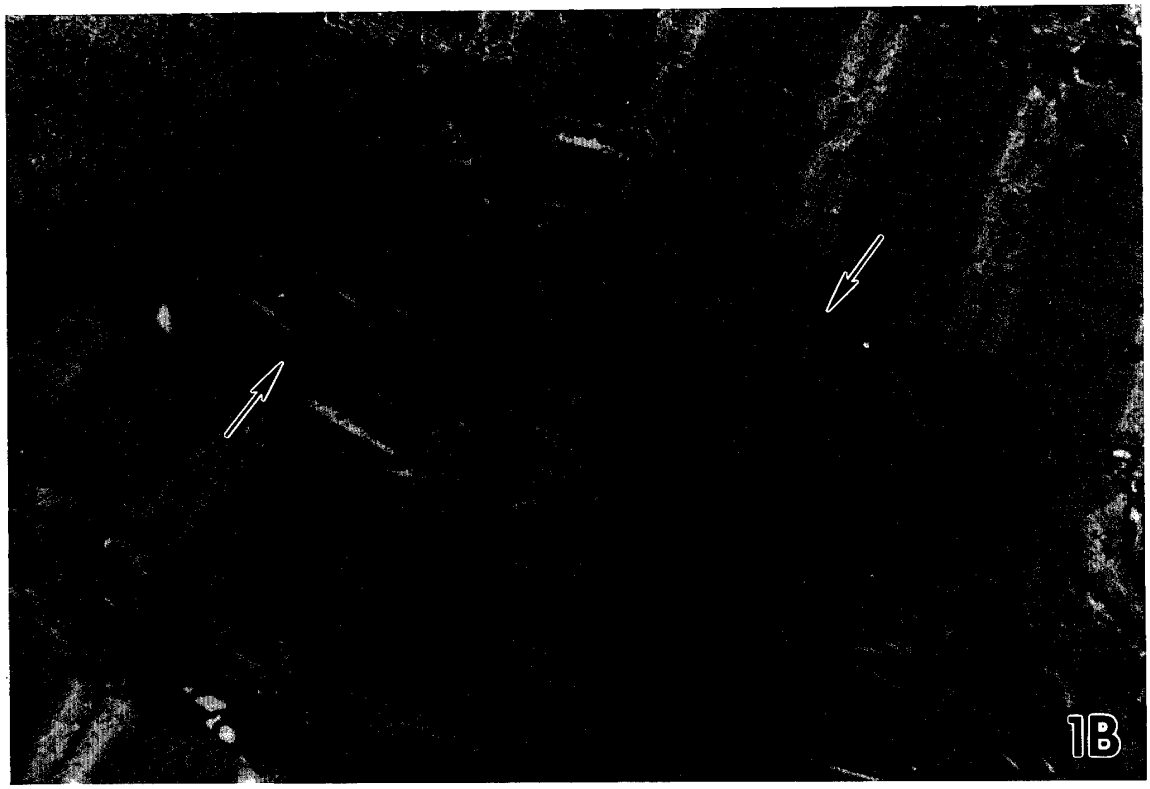
LEGENDS FOR MICROGRAPHS - HISTOPATHOLOGY - PILOT STUDY

Plate 1. Lesions in medaka with disseminated mycobacteriosis.

Figure A. Multiple granulomata in kidney of control medaka fed PC-diet. Granulomata fill intertubular space and cellular casts are in tubular lumina. H&E 250X.

Figure B. Electron micrograph of skeletal muscle in body wall of control medaka. Arrows point to bacteria which are dispersed between organelles of this myocyte displacing contractile elements. M- mitochondrion. TEM , 5,000 X.


1A

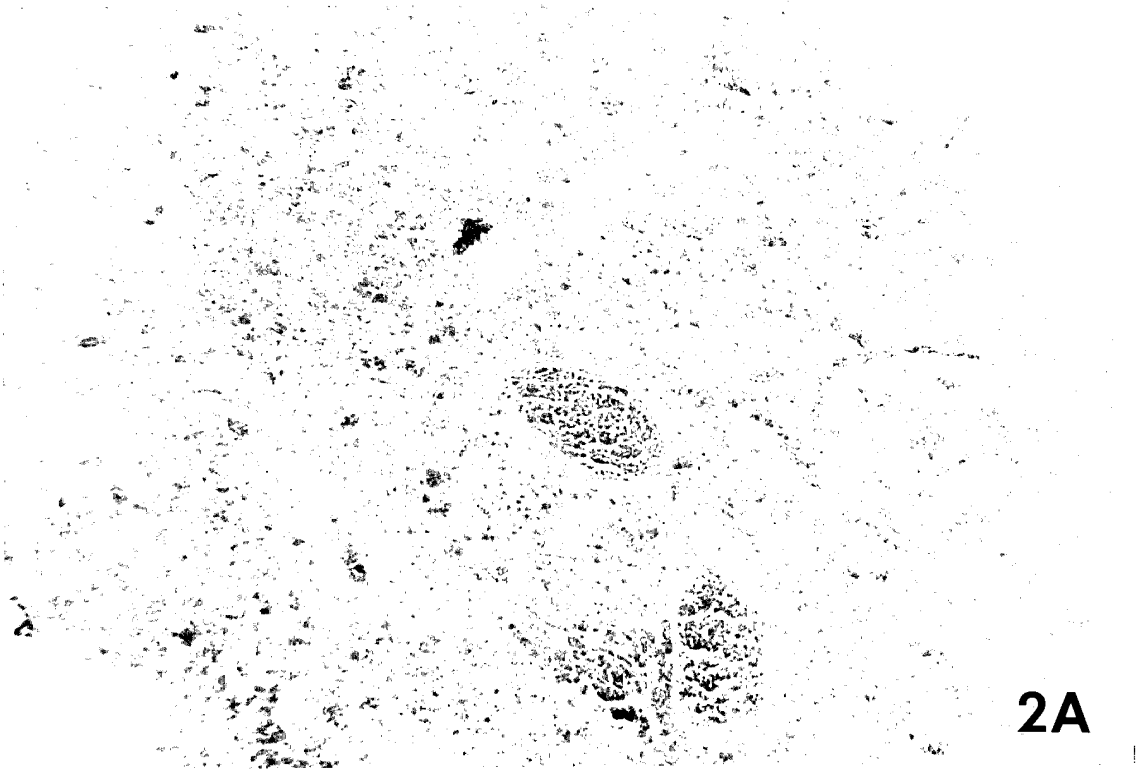

1B

LEGENDS FOR MICROGRAPHS - HISTOPATHOLOGY - PILOT STUDY

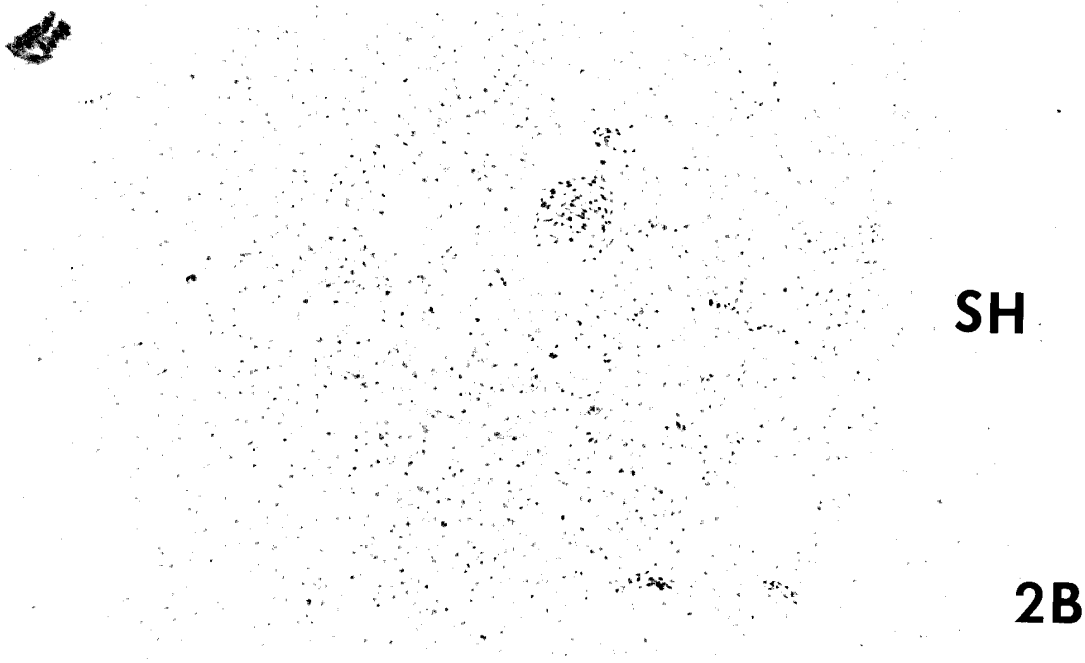
Plate 2. Lesions of fish exposed to AFB_1.

Figure A. Mosaic pattern of hepatic parenchyma in female medaka fed the F/A-diet and exposed to 3.00 ppm AFB_1. Alternating focal regions of basophilic cells contrast with other regions which show pronounced eosinophilia. This panhepatic response was associated with active AFB_1 exposure and, to a very limited extent, in controls fed F/A-diet.

Figure B. Female medaka exposed to 3.0 ppm AFB_1 and fed the F/A diet for 6 months. An advanced, large focus of cellular alteration contains cells with four phenotypes: amphophilic, basophilic, clear cell and eosinophilic is shown. Focal spongiosis hepatis (SH) is nearby. H&E X250.



2A



SH

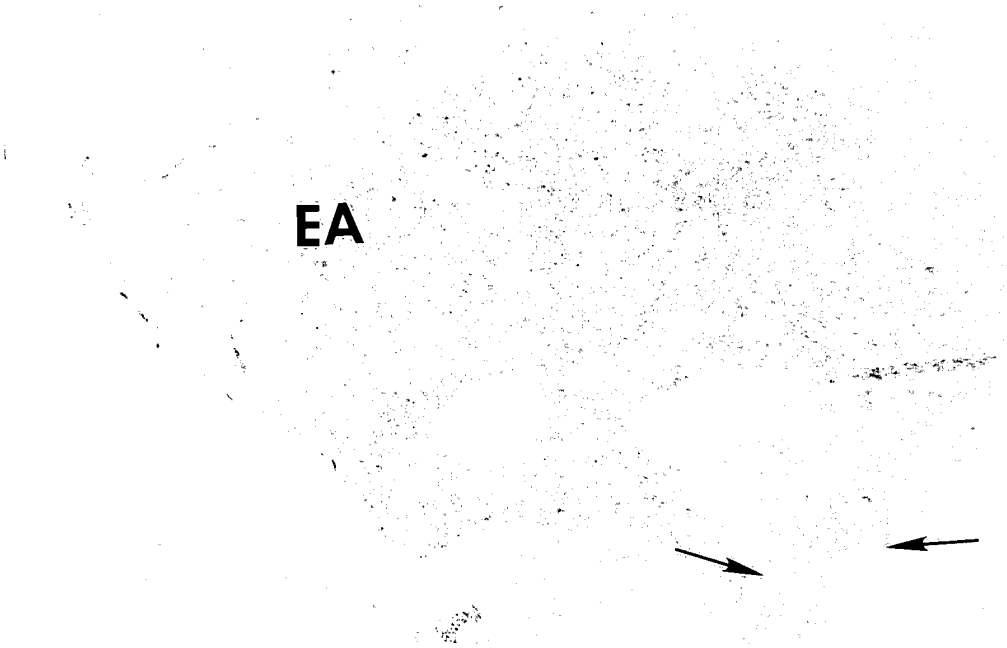
2B

LEGENDS FOR MICROGRAPHS - HISTOPATHOLOGY - PILOT STUDY

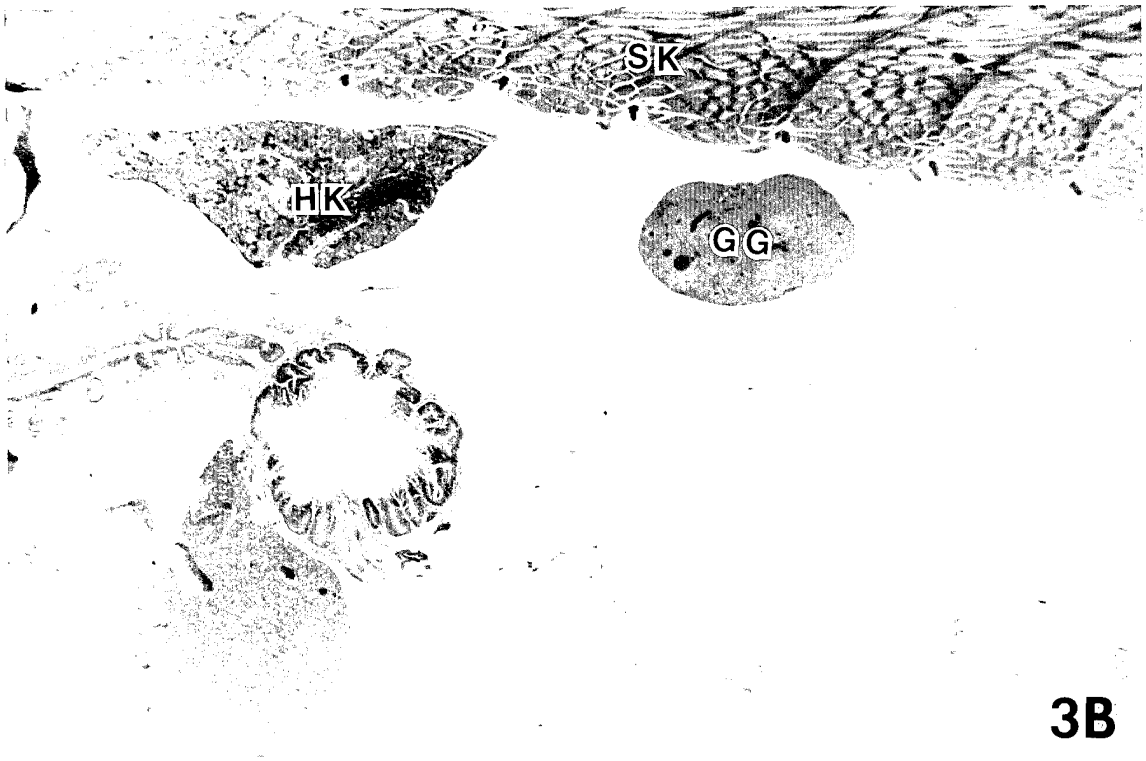
Plate 3. Adenomata in medaka exposed to AFB_1 .

Figure A. Female medaka exposed to 3.0 ppm AFB_1 in F/A-diet for 6 months. One eosinophilic adenoma (EA) and one basophilic focus (arrows) are shown in this liver section. H&E X 125.

Figure B. Male medaka exposed to 1.0 ppm AFB_1 in F/A-diet for 6 months. Skeletal muscle in body wall (SK) is shown at top of field. HK- head kidney. Gas gland (GG) contains adenoma making the structure resemble a solid organ. H&E X25.



3A



3B

RESULTS FOR DEFINITIVE AFB_1 STUDY

Background- The 1992 pilot study design employed aflatoxin B_1 (AFB_1) addition to the two diets, commercial flake with two days supplementation with Artemia sp. nauplii (FA) and the purified diet with casein base (PC). On those two days of each week that the FA groups were given brine shrimp nauplii, the corresponding PC group was fed PC-diet without AFB_1. As was stated above, the pilot study was conducted using static exposure conditions within a carcinogen approved glove box with dedicated air exhaust. Each tank was equipped with a biological filter suspended from the outer wall and water was continuously pumped over the filter and back into the tank. Prior to initiation of the definitive AFB_1 bioassay, a closed recirculating system with four, 75-liter volume tanks was built. To establish the function of the biological filter, 20 fish were housed in each tank and ammonia and nitrite levels were monitored during this period to ascertain the nitrifying potential of the filters for the recirculating system. Upon verification of function, the experimental fish were then moved to each tank and allowed to acclimate. Water chemistry was performed on all tanks for the first 12 days, then weekly for the duration of the study. Details of acceptable ranges for water quality values are found in Chapter 1 of this report.

Detailed aspects of the fixation, embedment, and processing of fish for paraffin section analysis are contained within Chapter 2 of this report. All procedures performed in Chapter 4 were identical to those described in the previous chapter.

Mortality in 1992 versus 1994 - For the 1994 AFB_1 study, a recycling system (see Methods above) was used and survival improved. Mortality in the 1992 pilot study and the 1994 definitive study are compared after 210 days of exposure (fig. 3-27). The 1994 control mortality was between 20 and 30% and was similar in both diets. The 1994 mortality for 3.0 ppm AFB_1 -

exposed fish showed PC-fed fish had a mortality of about 40% while FA-fed fish mortality was 60%.

Mortality is shown for the different experimental groups in Figures 3-28 through 3-31. Figure 3-28 illustrates the cumulative mortality for fish fed the F/A-diet. Those fish given the control F/A-diet had a total mortality over the 210 days of the study of 59 fish. During this same period of time, those receiving the 3.0 ppm addition of AFB_1 to the F/A-diet showed a mortality of 125 individuals (Figure 3-28). Cumulative mortality for fish fed the PC-diet is shown in Figure 3-29. Control fish, those receiving PC-diet only, showed a total mortality of 62 individuals. During this same period of time, those fish fed the above diet with addition of 3.0 ppm AFB_1 showed a cumulative mortality of 82 individuals. Greatest amount of mortality occurred in the interval between 150 and 210 days. Cumulative mortality is shown graphically for the two control groups using PC-diet and F/A-diets. These are illustrated in Figure 3-30. PC fed fish showed slightly greater mortality during the earlier period of the test than did their FA fed cohorts. Total cumulative mortality for the two control groups was similar over the study. A total of 62 mortalities was encountered in PC-fed control group while 55 mortalities occurred in the corresponding FA-fed control group. Figure 3-31 illustrates cumulative mortality comparing the AFB_1 (3.0 ppm) exposed fish given either PC or F/A-diet. At first, in the interval between the initiation of exposure and the first 140 days, mortality was higher in the PC-diet group. Between 100 and 150 days, the mortality curve for the FA fed fish crossed over that of their PC-fed cohorts. This resulted in a cumulative mortality of 125 individuals in the F/A-diet group while the PC fed group contained approximately 80 mortalities.

Statistical Testing for Survival - To test effect of the various treatments on survival, we

applied two well-known statistics (log-rank and Wilcoxon). Tests focused on homogeneity of survival curves for the various treatments and a summary of the four comparisons is found in Table 3-7. Over the 7 months, the percent survival (% censored) represented the percentage of 200 fish that lived to the end of the test (217 days). For example, for the FA 0.0 ppm AFB_1 group, 200 started the test, 59 failed (died) 141 were censored (survived) and the % Censored was 70.5. Remaining groups and their respective numbers for each category are presented in Table 3-7.

Within the FA-fed groups, the effect of 3.00 ppm AFB_1 on survival of medaka was tested (Table 3-7). Whereas 59 mortalities occurred in controls, 125 were seen in 3.0 ppm AFB_1 - exposed medaka. The percent medaka surviving (% censored) the tests was 70.5 (0.0 ppm AFB_1) and 37.5 (3.0 ppm AFB_1) respectively. Addition of 3.0 ppm AFB_1 to F/A-diet significantly decreased ($P \leq 0.001$) survival.

Within the PC-fed groups, the effect of 3.0 ppm AFB_1 on survival of medaka was tested (Table 3-7). Whereas 62 mortalities occurred in controls, 82 occurred in 3.0 ppm AFB_1 - exposed medaka. The percent medaka surviving (% censored) the tests was 69.0 (0.0 ppm AFB_1) and 59.0 (3.0 ppm AFB_1). Addition of 3.0 ppm AFB_1 to PC-diet significantly decreased ($P \leq 0.05$) survival.

Survival was also compared between medaka fed FA containing 3.0 ppm AFB_1 and medaka fed identical levels of AFB_1 in PC-diet. Results are summarized in Table 3-7. Percent survivors (% censored) for FA fed and AFB_1 exposed medaka was 37.5 compared to 59.0 for PC fed and AFB_1 exposed medaka. Survival was significantly ($P \leq 0.001$) less in FA - versus PC - fed medaka.

Table 3-7. Statistical Analysis of Effects of Various Treatment on Survival (N_{TOT}) of Medaka

Group	N_{TOT}	N_{failed}	$N_{survived}$	% Survivor
FA 0.0 ppm	200	59	141	70.5
FA 3.0 ppm	200	125	75	37.5
PC 0.0 ppm	200	62	138	69.0
PC 3.0 ppm	200	82	118	59.0

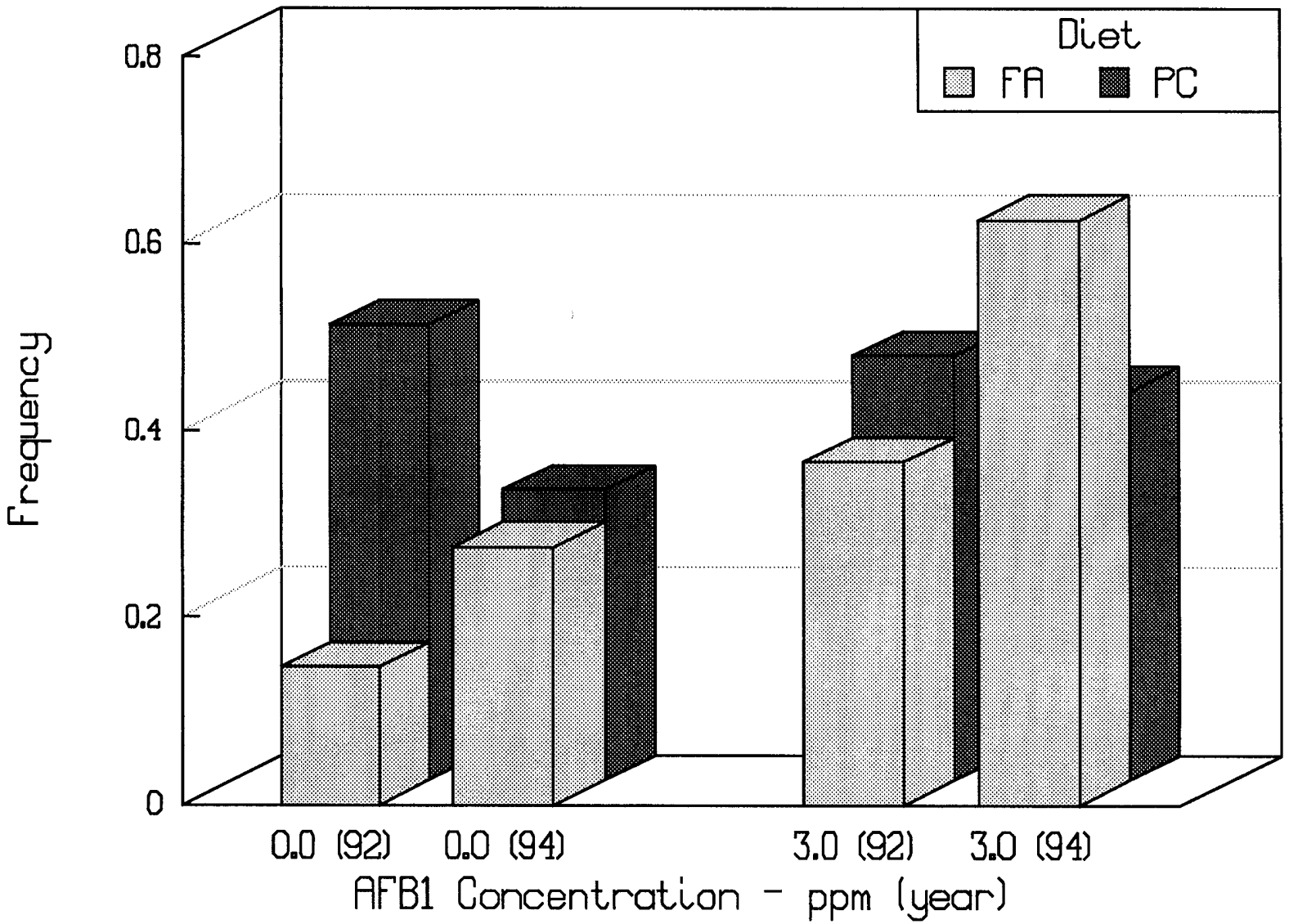
Comparisons	Log rank (P-values)	Wilcoxon (P-values)
FA 0.0 vs FA 3.0 ppm	0.0001*	0.0001*
FA 0.0 vs PC 0.0 ppm	0.6499	0.5980
PC 0.0 vs PC 3.0 ppm	0.0240	0.0184
PC 3.0 vs FA 3.0 ppm	0.0002*	0.0009*

% Survival was the percentage of 200 fish that lived to the end of the experiment (217 days). Log rank and Wilcoxon test were applied to test the homogeneity of survival curves over various treatments. * = Statistically significant between group.

Comparisons of survival distributions by diet and AFB_1 concentration are shown graphically in figure 3-32. From six months on, the FA-fed, AFB_1 - exposed group showed marked difference in survival distribution from the other three groups. The survival distributions for the PC-fed and FA-fed controls were similar. PC-fed, AFB_1-exposed group showed an intermediate distribution.

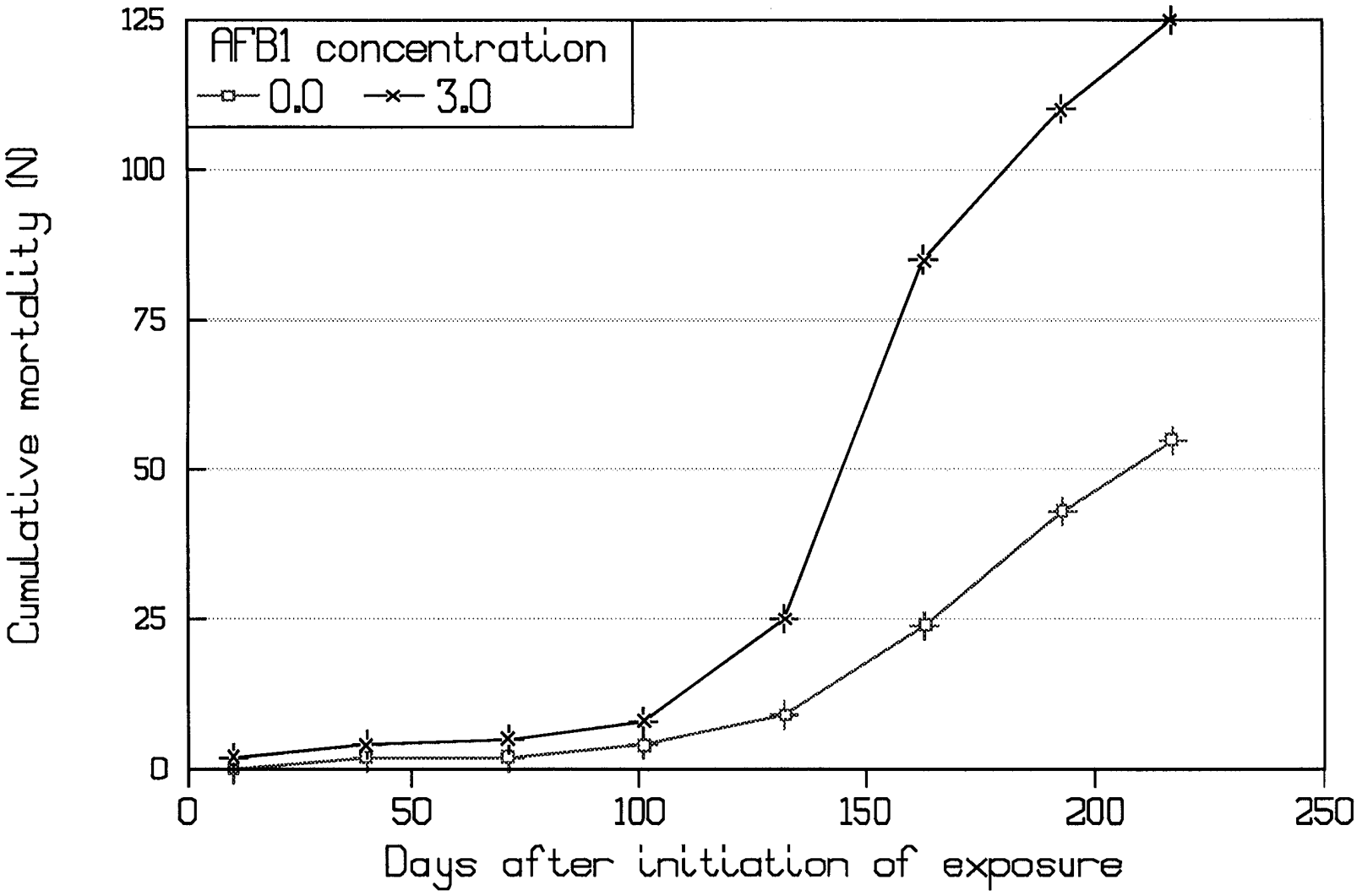
LEGENDS FOR FIGURES

Figure 3-27. Mortality of 1992 (pilot) versus 1994 (definitive) study


Diet
FA
PC
0.8
0.6
0.4
0.2
0
Frequency
0.0 (92)
0.0 (94)
3.0 (92)
3.0 (94)
AFB1 Concentration - ppm (year)

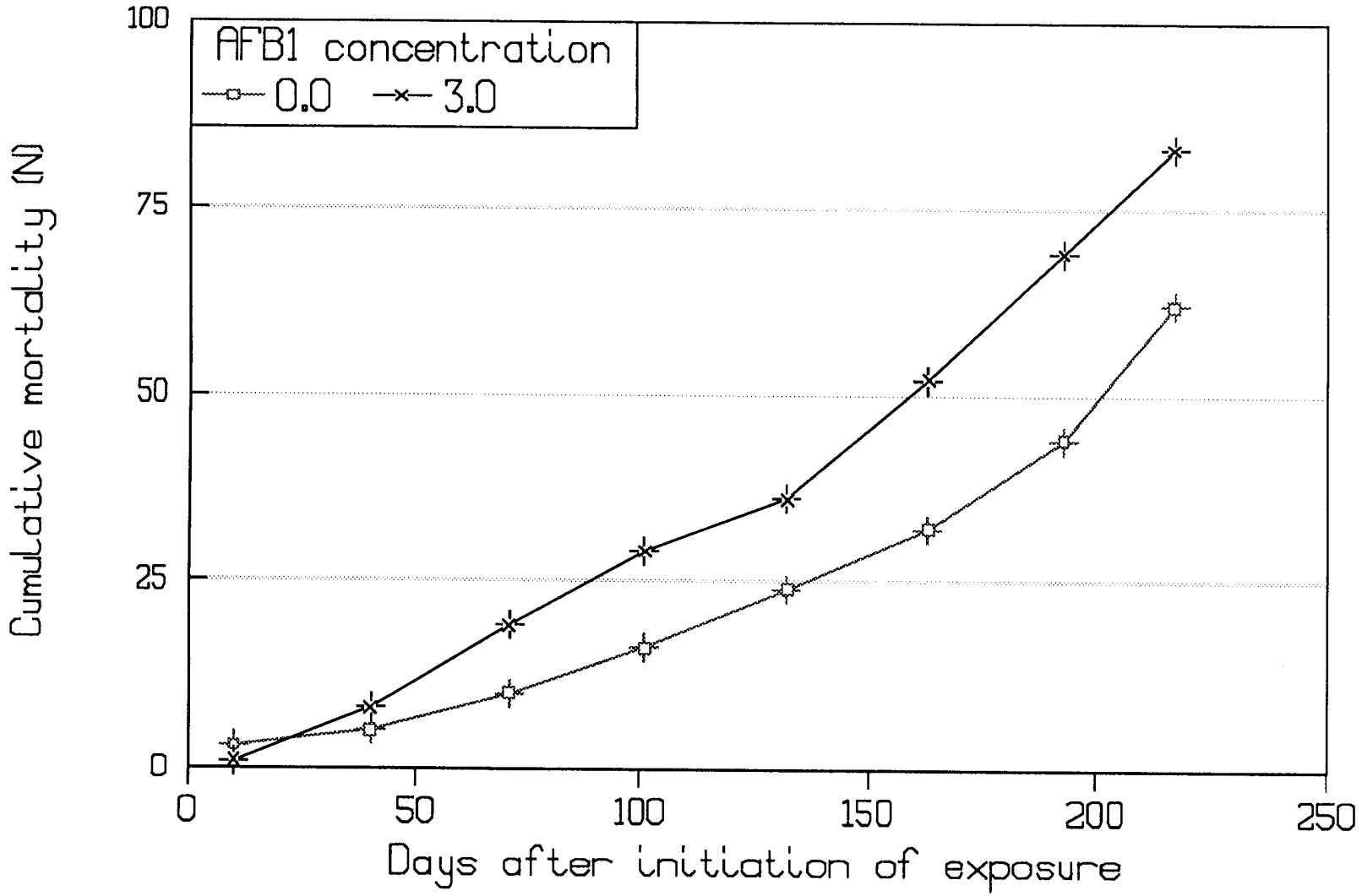
LEGENDS FOR FIGURES

Figure 3-28. Cumulative mortality in AFB_1-exposed medaka fed F/A diet.


AFB1 concentration
0.0
3.0
Cumulative mortality (N)
125
100
75
50
25
0
0
50
100
150
200
250
Days after initiation of exposure

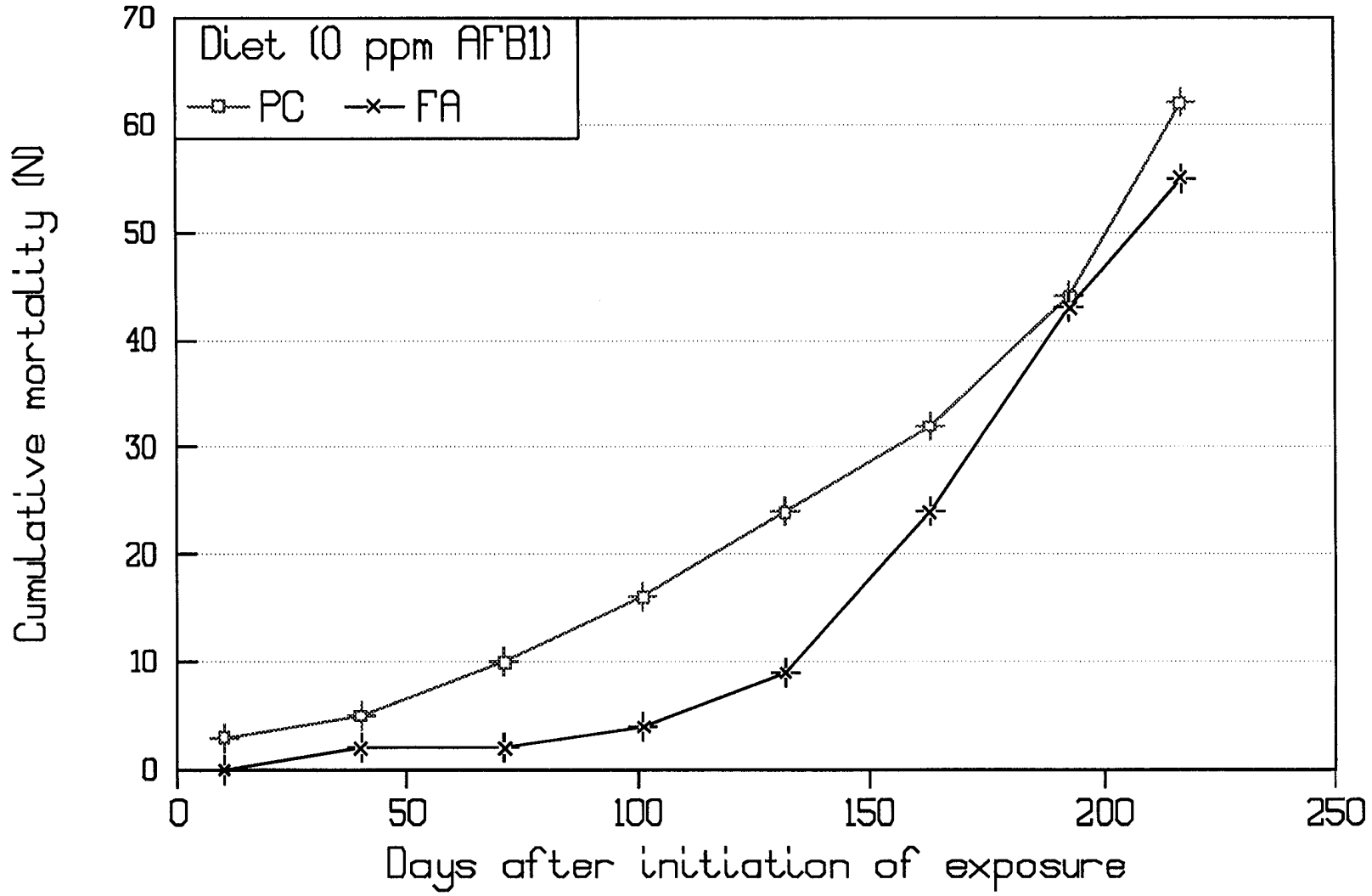
LEGENDS FOR FIGURES

Figure 3-29. Cumulative mortality in AFB_1-exposed medaka fed PC-diet.


AFB1 concentration
0.0
3.0
Cumulative mortality (N)
100
75
50
25
0
0
50
100
150
200
250
Days after initiation of exposure

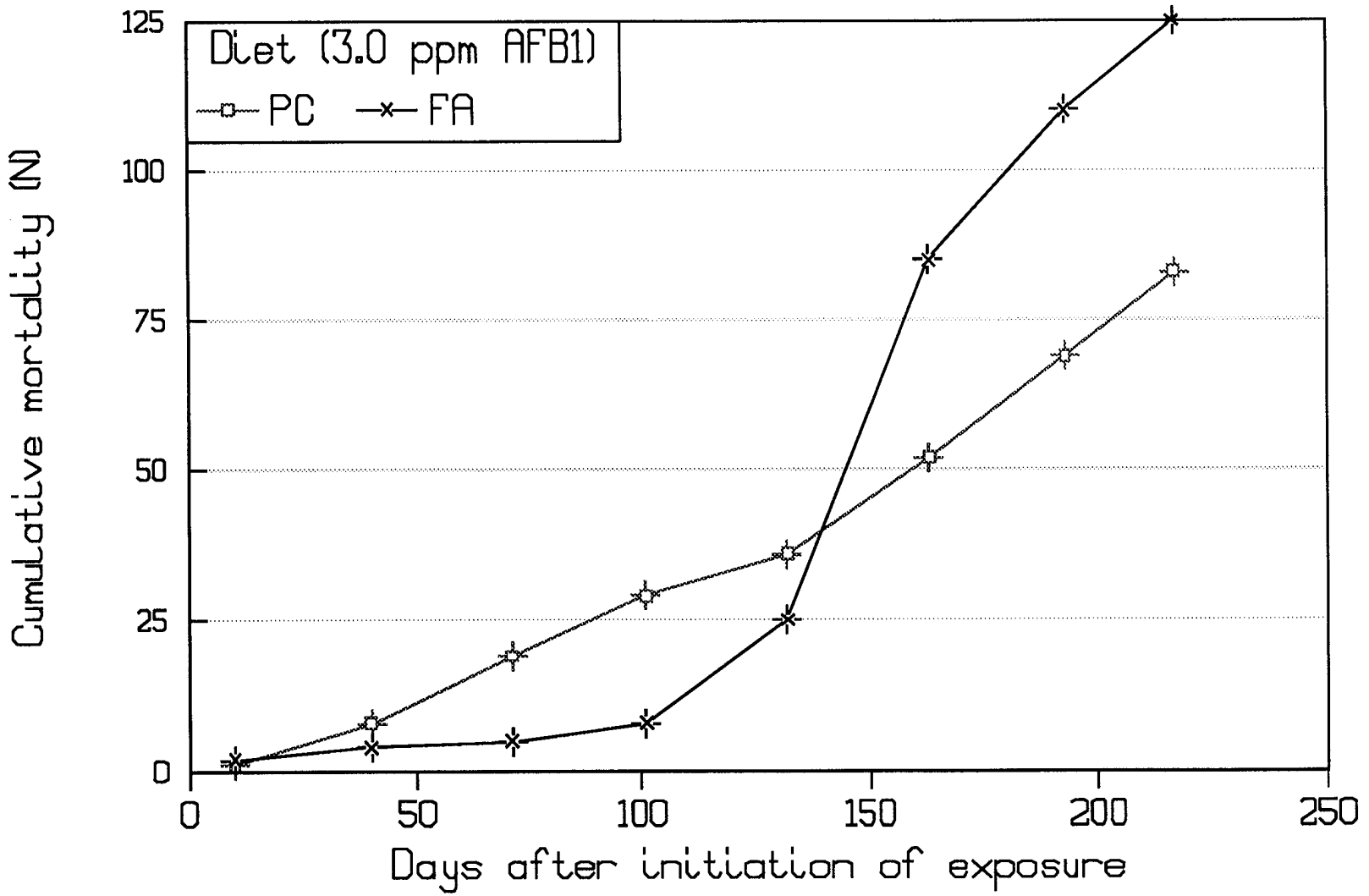
LEGENDS FOR FIGURES

Figure 3-30. Cumulative mortality in medaka controls fed F/A or PC-diets.


Diet (0 ppm AFB1)
PC
FA
Cumulative mortality (N)
70
60
50
40
30
20
10
0
0
50
100
150
200
250
Days after initiation of exposure

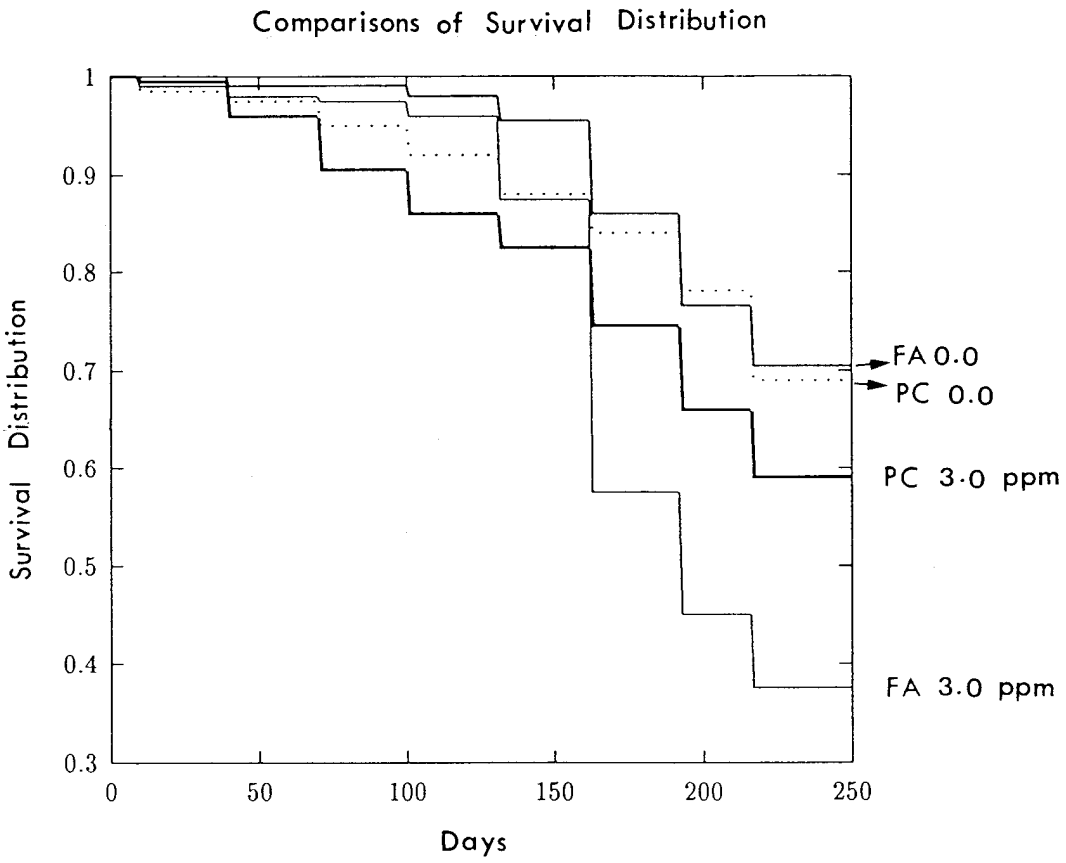
LEGENDS FOR FIGURES

Figure 3-31. Cumulative mortality in medaka exposed to 3.0 ppm AFB_1 and fed F/A or PC-diets.


Diet (3.0 ppm AFB1)
PC
FA
Cumulative mortality (N)
125
100
75
50
25
0
0
50
100
150
200
250
Days after initiation of exposure

LEGENDS FOR FIGURES

Figure 3-32. Comparisons of survival distributions by diet and AFB_1 concentrations.


Comparisons of Survival Distribution
Survival Distribution
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
50
100
150
200
250
Days
FA 0.0
PC 0.0
PC 3.0 ppm
FA 3.0 ppm

Histopathology - Three month sampling, Definitive study

A total of 80 fish, 20 from each of the four experimental groups were examined and processed for paraffin embedment. The results are reported in Table 3-8. Fish fed either diet and exposed to AFB_1 showed panhepatic toxicity with alternating focal regions of eosinophilia and basophilia. This lesion, signifying hepatotoxicity, was at higher frequency in females of the study regardless of diet. Fish fed the F/A-diet containing AFB_1 showed a 25% incidence of hepatic neoplasms. Of the 5 tumor-bearing fish, 4 were female and 1 was male. Fish fed the PC-diet showed a lower frequency of hepatic neoplasms (10%). Of the tumor-bearing, PC-fed fish, 1 was a male and 1 was a female. FCA with basophilic and with eosinophilic phenotypes were encountered (Table 3-8). A marked gender specific frequency was observed (greater in females) in both dietary groups. Eight of the 20 fish from the FA group showed FCA (40% frequency). Of these FCA-positive fish, 6 were females and 2 males. For fish fed the PC-diet, a frequency of 25 % was encountered. Of these foci-bearing fish, 4 were females and 1 male. These results suggest that initiation of FCA and the promotion from FCA to tumor occurs more rapidly in fish fed the F/A-diet and that females have a higher frequency of lesions at this earlier stage than do males.

Histopathology Results - Six Months

A total of 80 fish were sampled after six months dietary AFB_1 exposure in the definitive study. The frequency of occurrence of panhepatic toxicity, FCA and tumors is summarized in table 3-9. The panhepatic toxicity, mosaic pattern was seen in livers FA-fed and carcinogen exposed fish at 30% mean frequency with higher % in females (50%). A total of five out of eight females fed the PC-diet and exposed to AFB_1 showed panhepatic toxicity (63% frequency).

Table 3-8 Frequency of Occurrence of Panhepatic Toxicity, FCA, and Tumor in Medaka Exposed to Dietary AFB_1 for Three Months

	Sex	Toxicity	Basophilic	Clear Cell	Eosinophilic	Tumors
F/A diet	F	4/10	6/10	0/10	1/10	4/10
	M	2/10	2/10	0/10	0/10	1/10
PC-diet	F	8/14	4/14	0/14	1/14	1/14
	M	0/6	0/6	0/6	0/6	1/6

N = 20 fish per group. Since gender was not determined until histopathology, number (N) of males and females vary within groups.

Table 3-9 Frequency of Occurrence of Panhepatic Toxicity, FCA and Tumor in Medaka Exposed to Dietary AFB_1 for Six Months

	Sex	Toxicity	Basophilic	Clear Cell	Eosinophilic	Tumors
F/A diet	F	3/6	3/6	0/6	2/6	6/6
	M	3/14	2/14	3/14	1/14	12/14
PC-diet	F	5/8	4/8	3/8	2/8	6/8
	M	5/12	5/12	1/12	3/12	3/12

N = 20 fish per group. Since gender was not determined until histopathology, number (N) of males and females vary within groups.

Their male cohorts showed 42% frequency for the same condition. Basophilic FCA frequency in the FA-fed, AFB_1-exposed medaka was 25% with higher frequency in females. In PC-fed and AFB_1-exposed medaka, basophilic FCA frequency was 45% with higher frequency in females. Eosinophilic FCA were less common in either dietary group. No sharp gender differences in frequency for this phenotype were noted. Clear cell FCA, a phenotype not encountered after 3 months exposure, were seen in three of 20 medaka of the FA group and in 4 of 20 medaka of the PC group. Frequency for hepatic neoplasms was 90% in FA-fed medaka exposed to AFB_1; again, higher frequency was seen in females. Frequency for the same condition was 45% in PC-fed and carcinogen exposed medaka with highest frequency in female fish (Table 3-9).

Histopathology Results - Seven Months

The frequency of occurrence of FCA with the basophilic phenotype is shown in Figure 3-33. None of the male controls possessed foci. Histopathology results for females indicated that 2 of the medaka fed the F/A-diet with 0.0 ppm AFB_1, but none of the PC-fed females, had basophilic foci (Figure 3-33). In male medaka exposed to 3.0 ppm AFB_1, a higher frequency of occurrence of basophilic foci was noted in the fish fed the PC-diet. When females were examined, 40% of those fed the F/A-diet and exposed to the carcinogen showed basophilic foci. A much lower occurrence of foci was noted in females fed the PC-diet.

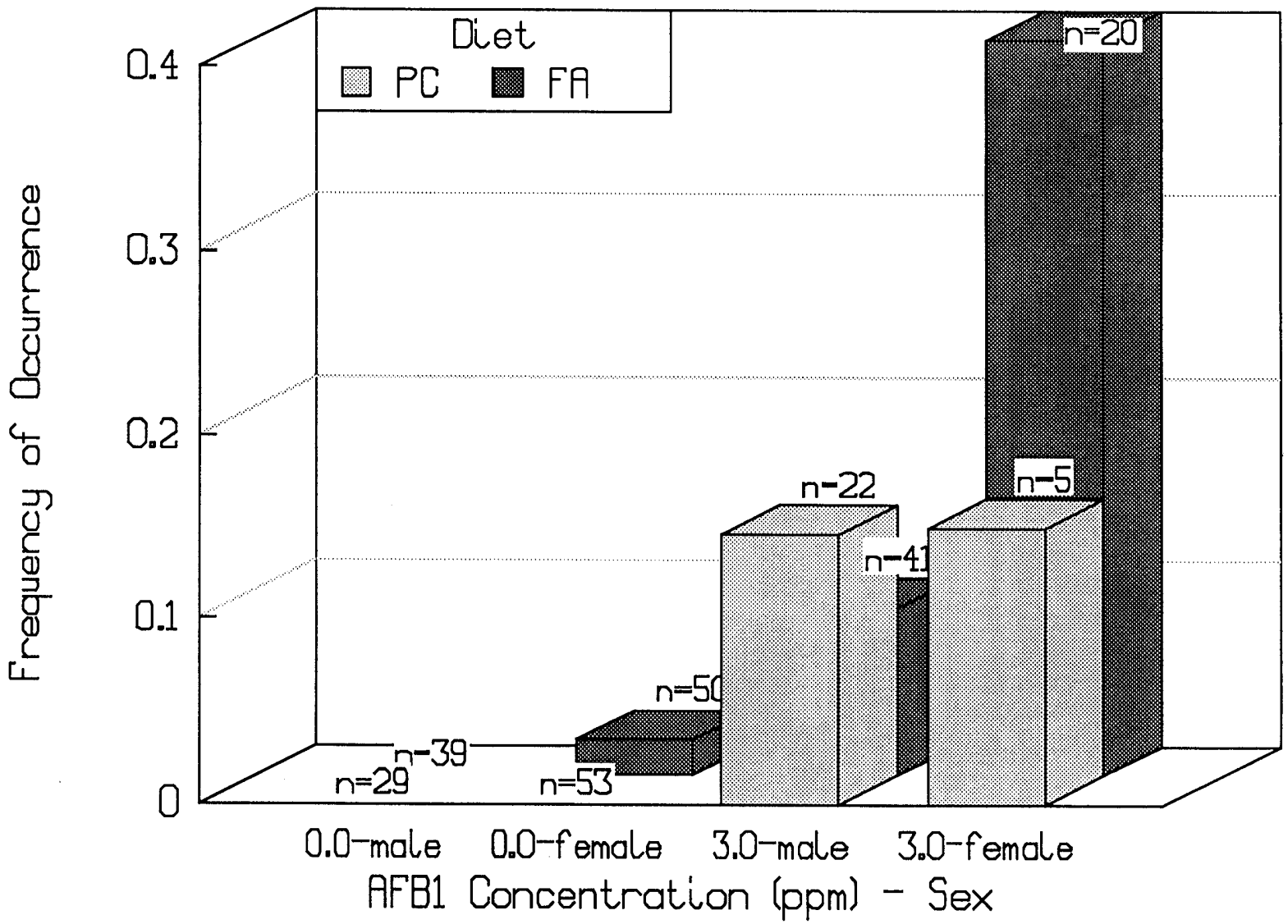
Results for eosinophilic FCA phenotypes are illustrated in Figure 3-34. In the controls, 1 of the PC fed medaka showed an eosinophilic focus. None of the FA fed control medaka were positive for eosinophilic foci at seven months. When the medaka exposed to the carcinogen were analyzed, a higher incidence for eosinophilic foci characterized the PC fed fish versus their FA fed cohorts. Results for clear cell foci are illustrated in 3-35. Only six fish from the seven

month sampling showed clear cell foci. These were all found within males that were exposed to the carcinogen and fed the PC-diet. Results for amphophilic foci are illustrated in Figure 3-36. Both exposed PC-fed medaka and their FA-fed cohorts showed amphophilic foci, but at very low frequency of occurrence.

Figures 3-37 and 3-38 illustrate frequency of occurrence for hepatocellular neoplasms. One eosinophilic adenoma was encountered in 86 control medaka fed the F/A-diet. None of the PC-fed control medaka showed hepatic neoplasms. When the fish exposed to the carcinogen were analyzed, approximately 50% of 66 PC-fed fish had neoplasms. A total of 29 medaka fed the AFB_1 containing F/A-diet were analyzed for presence of hepatic neoplasms. Among these fish, a frequency of occurrence of 77% was observed. Figure 3-38 presents gender specific information on frequency of hepatic neoplasms. The only neoplasm encountered in controls of the study was seen in a single medaka fed the F/A-diet. This lesion was a hepatic adenoma. In male fish exposed to the carcinogen, F/A-dietary group showed higher frequency (80 versus 55%). Females also showed higher frequency in the FA-fed and carcinogen-exposed group (80 versus 57%).

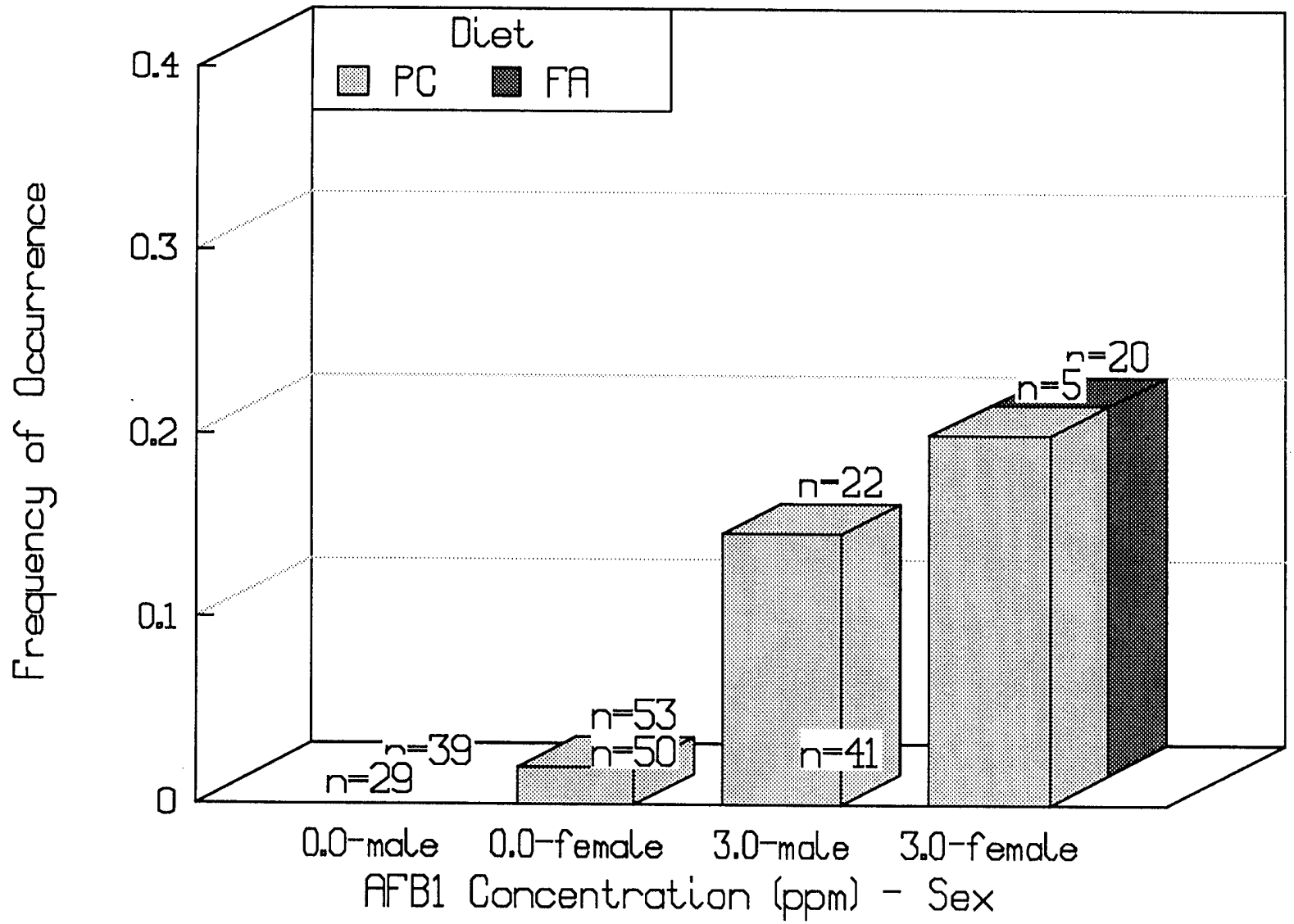
LEGENDS FOR FIGURES

Figure 3-33. Effect of diet and gender on frequency of occurrence of basophilic foci in medaka liver. 0.0 and 3.0 ppm = AFB_1 concentration in diet. Number (N) is provided for each gender and exposure concentration for each diet (PC and F/A). Medaka were exposed to AFB_1 in diet for six months and then allowed to recover for one additional month.


Diet
PC
FA
Frequency of Occurrence
0.4
0.3
0.2
0.1
0
n=29
n=39
n=53
n=50
n=22
n=41
n=5
n=20
0.0-male
0.0-female
3.0-male
3.0-female
AFB1 Concentration (ppm) - Sex

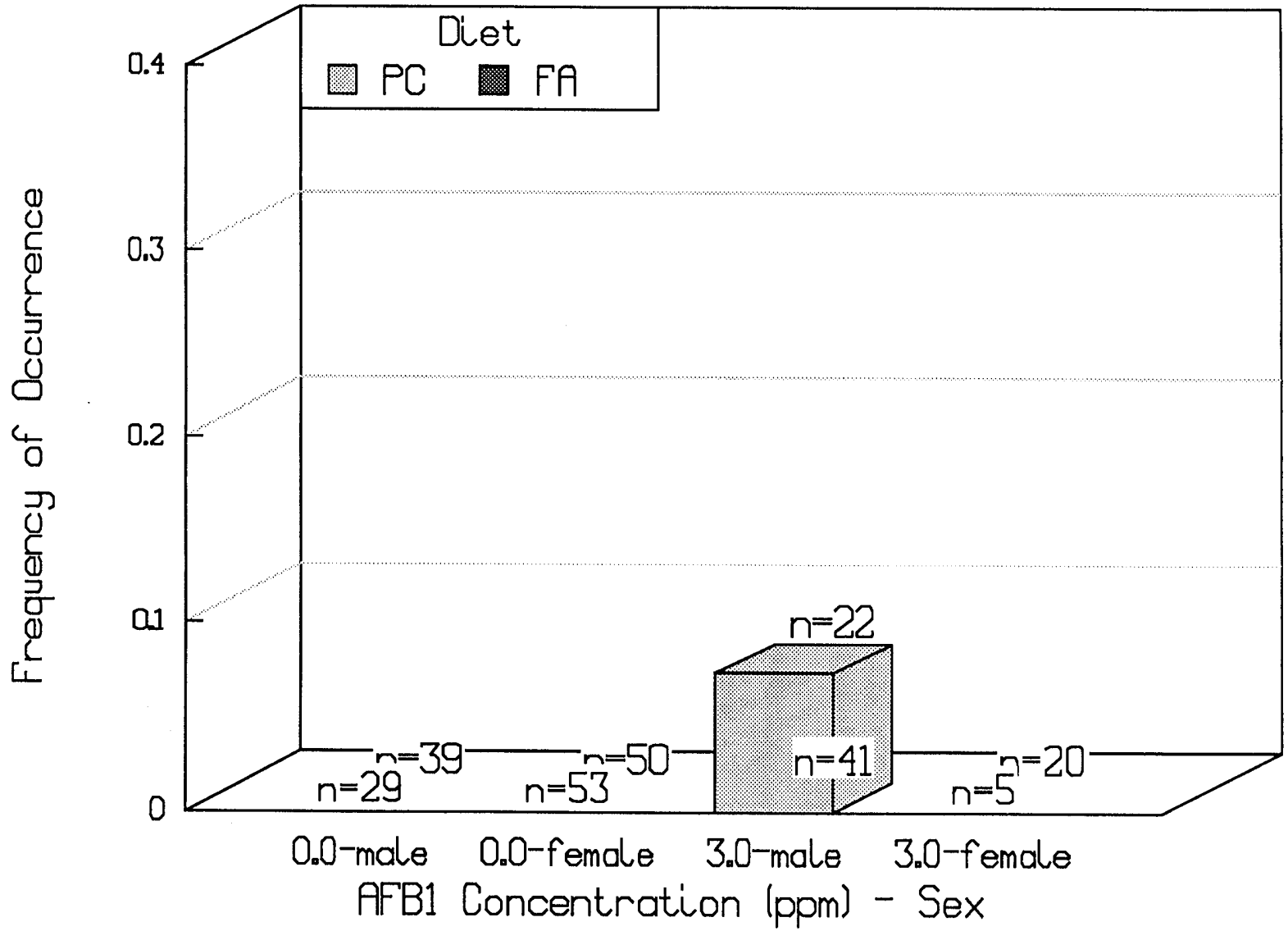
LEGENDS FOR FIGURES

Figure 3-34. Effect of diet and gender on frequency of occurrence of eosinophilic foci in medaka liver. 0.0 and 3.0 ppm = AFB_1 concentration in diet. Number (N) is provided for each gender and exposure concentration for each diet (PC and F/A). Medaka were exposed to AFB_1 in diet for six months and then allowed to recover for one additional month.


Diet
PC
FA
0.4
0.3
0.2
0.1
0
Frequency of Occurrence
n=29
n=39
n=53
n=50
n=22
n=41
n=5
n=20
0.0-male
0.0-female
3.0-male
3.0-female
AFB1 Concentration (ppm) - Sex

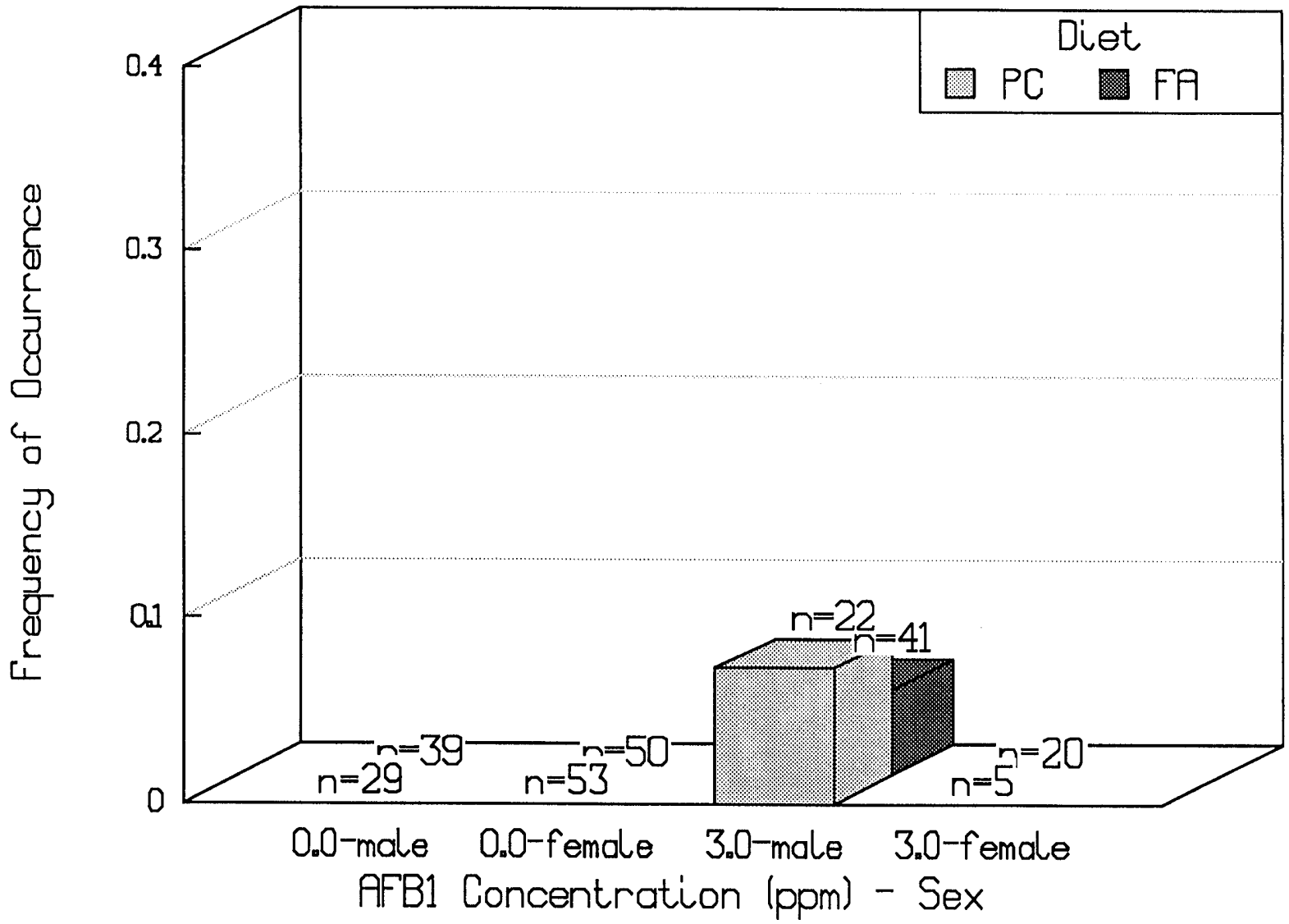
LEGENDS FOR FIGURES

Figure 3-35. Effect of diet and gender on frequency of occurrence of clear cell foci in medaka liver. 0.0 and 3.0 ppm = AFB_1 concentration in diet. Number (N) is provided for each gender and exposure concentration for each diet (PC and F/A). Medaka were exposed to AFB_1 in diet for six months and then allowed to recover for one additional month.


Diet
PC
FA
Frequency of Occurrence
0.4
0.3
0.2
0.1
0
n=22
n=39
n=50
n=29
n=53
n=41
n=20
n=5
0.0-male
0.0-female
3.0-male
3.0-female
AFB1 Concentration (ppm) - Sex

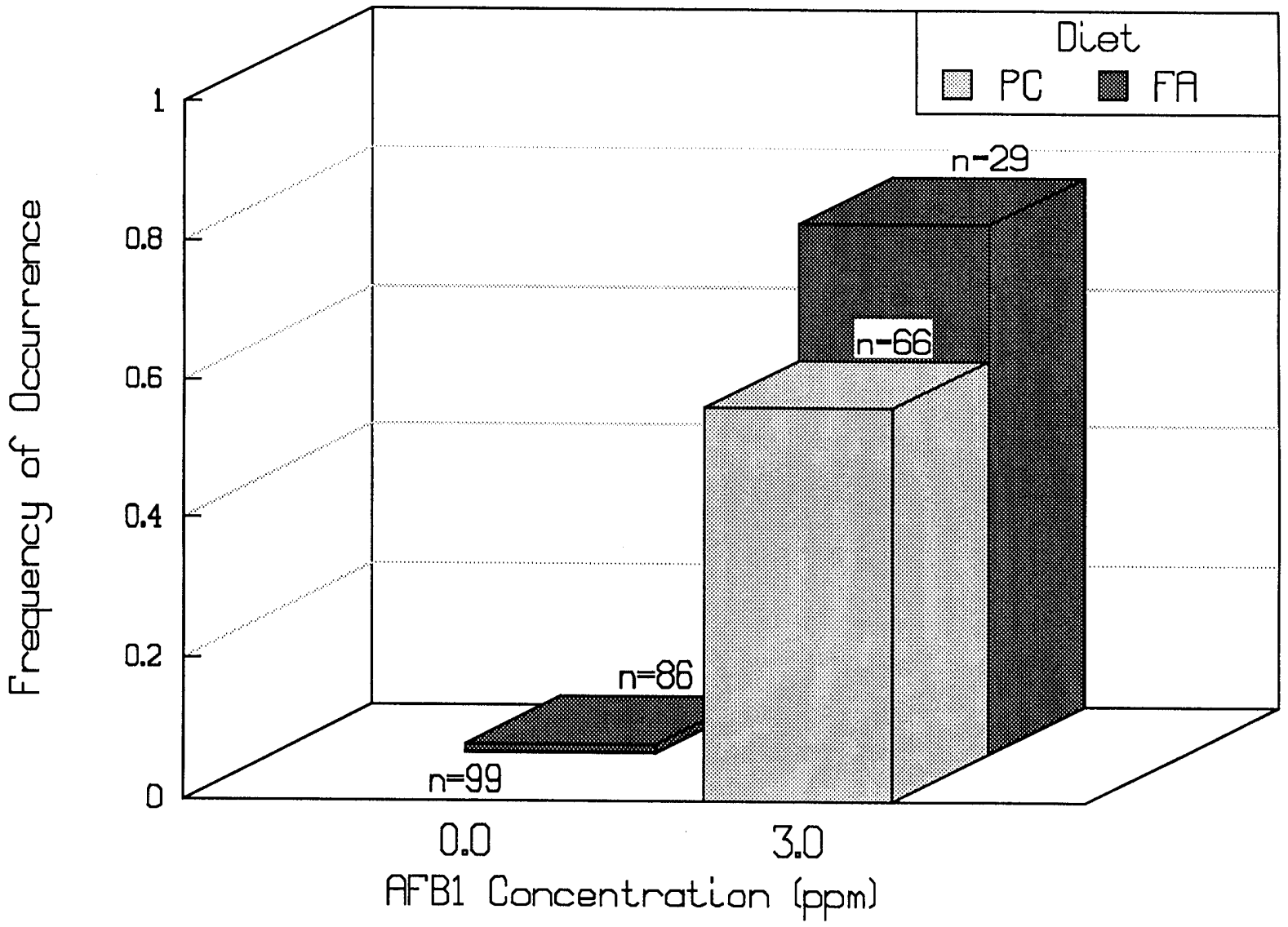
LEGENDS FOR FIGURES

Figure 3-36. Effect of diet and gender on frequency of occurrence on amphophilic foci in medaka liver. These frequencies were not separated by gender.


Diet
PC
FA
Frequency of Occurrence
0.4
0.3
0.2
0.1
0
n=29
n=39
n=53
n=50
n=22
n=41
n=5
n=20
0.0-male
0.0-female
3.0-male
3.0-female
AFB1 Concentration (ppm) - Sex

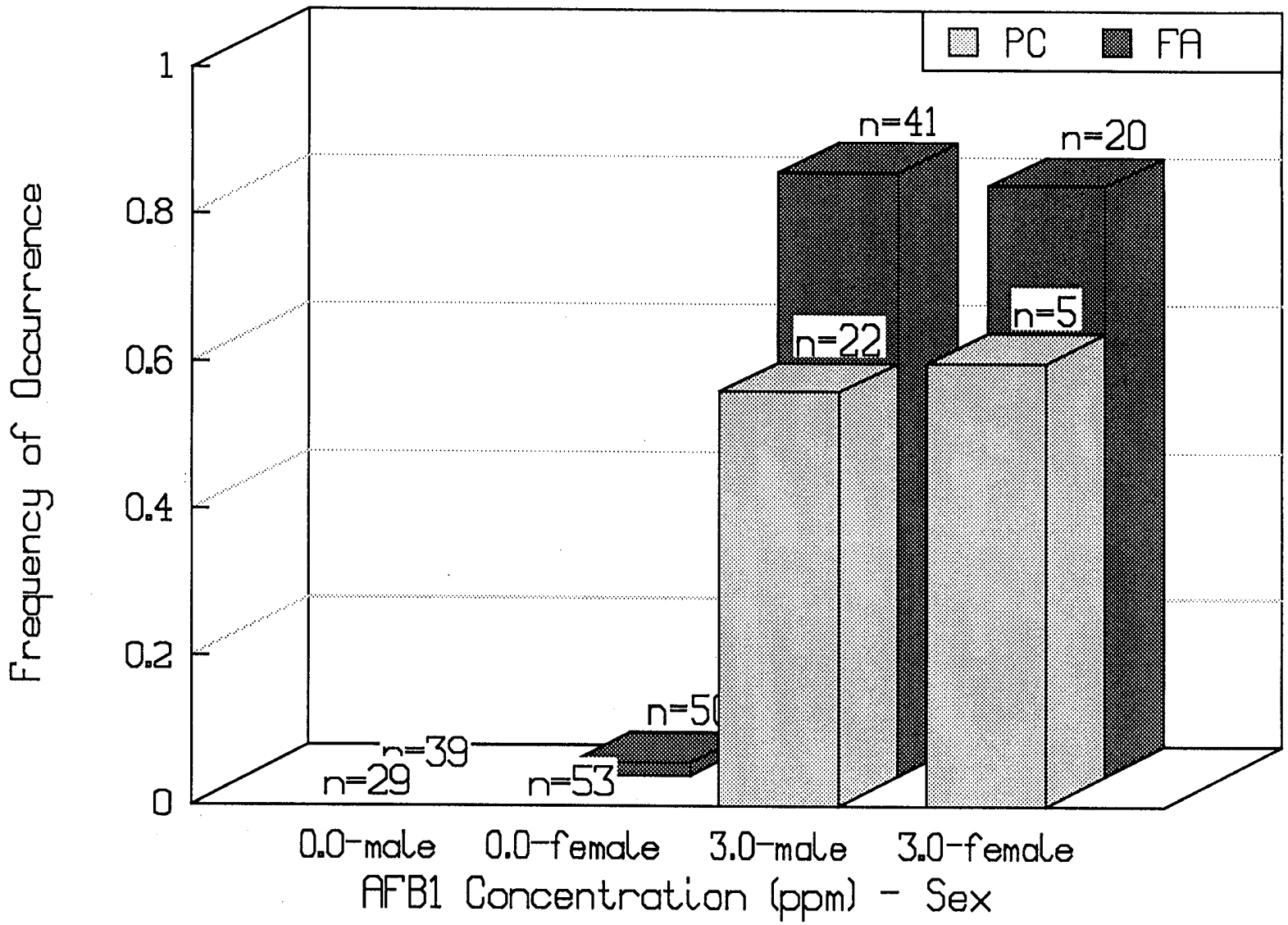
LEGENDS FOR FIGURES

Figure 3-37. Frequency of hepatic neoplasms as a function of diet in medaka exposed to AFB_1. All fish were exposed to either 0.0 (control) or 3.0 ppm AFB_1. Fish were fed either the PC or F/A diet. Exposure to AFB_1 was for six months followed by one month recovery.


Diet
PC
FA
Frequency of Occurrence
1
0.8
0.6
0.4
0.2
0
n=29
n=66
n=86
n=99
0.0
3.0
AFB1 Concentration (ppm)

LEGENDS FOR FIGURES

Figure 3-38. Frequency of occurrence of hepatic neoplasms by gender. All of the conditions of treatment of animals from which these data were derived were identical to that presented in figure legend for figure 3-37.


PC
FA
Frequency of Occurrence
1
0.8
0.6
0.4
0.2
0
n=41
n=20
n=22
n=5
n=39
n=29
n=53
0.0-male
0.0-female
3.0-male
3.0-female
AFB1 Concentration (ppm) - Sex

Histopathology of Mortalities - 1994 Definitive Test

Twice daily monitoring Monday through Friday and once daily on Saturday and Sunday of control and carcinogen treated fish was initiated on March 21, 1994 and continued until October 24, 1994 (the day at which the remaining fish were sacrificed). During the interval, 3/21/94 through 10/24/94, 59 fish fed the control F/A-diet died. Out of 59 fish, only 30 showed adequate fixation for subsequent histopathological analysis. Twenty-nine fish were either not fixed or autolysis had proceeded to an extent that histopathological analysis was deemed impossible. When examined histologically, no tumors were detected. Gonadal analysis confirmed gender for 23 of 30 fish. The number of females, males and "sex not determined" is shown in Table 3-10. A total of 125 fish fed the F/A-diet containing 3.0 ppm AFB_1 died and of these, 67 (44%) were examined histologically (Table 3-10). The remainder were too autolyzed to be examined by histology. Of the 67, 60 (32 females and 28 males) were shown to have hepatic neoplasms (89% of those examined). Of the female mortalities examined histologically (97% showed hepatic neoplasms). A total of 31 males fed the 3.0 ppm AFB_1 in F/A-diet died and were examined histologically. In these, 28 were tumor bearing (93%). No hepatic neoplasms were seen in one female, in three males, and in three fish which were classified as "sex not determined" (Table 3-10).

Similarly, 62 PC-fed control medaka died and of these, only 16 fish were fixed in formalin for histopathological analysis. A total of 46 fish were not fixed or were considered too autolyzed to warrant subsequent examination (Table 3-10). Seven were males, 7 were females, and 2 were "sex not determined" (Table 3-10). No hepatic neoplasms were found in the PC fed control group (Table 3-10). During the same interval, 82 fish fed the PC-diet containing 3.0 ppm AFB_1

Table 3-10. Tumor Analysis in Medaka Dying During 1994 Bioassay: Effect of Gender and Dietary Treatment

DIET		TUMOR BEARING FISH			TUMOR-FREE FISH			NUMBER OF FISH EXAMINED
PC		Female	Male	Unknown	Female	Male	Unknown	
	Control	0	0	0	7	7	2	16
	AFB_1 (3.0 ppm)	22	5	8	3	3	5	46
F/A	Control	0	0	0	11	12	7	30
	AFB_1 (3.0 ppm)	32	28	0	1	3	3	67

died and of these, 46 (56%) were examined histologically (Table 3-10). A total of 25 females were examined and 22 were positive for hepatic neoplasms (88%). A total of eight males were included in the population of PC fed fish dying during the test and examined histologically. Of the eight male medaka, 5 were positive (62.5%). Despite analysis of step sections through carcasses, gonadal tissue was not present in material examined for 13 of the PC-fed, AFB_1-exposed medaka dying during the test. Eight of the 13 fish were positive for hepatic neoplasms (61.5%) and five were negative.

Representative Histopathologic Alterations - AFB_1 Definitive Study

Disseminated mycobacteriosis (DM) was encountered in controls of this study fed either diet. The most severe cases appeared as multiple granulomata in skeletal muscle of body wall (pictured above) heart, liver, kidney other viscera and mesenteries. A representative section through liver, intestine, mesentery, and heart is shown (Plate 4, figure A). This fish was 10 months of age at the time of necropsy. Other than DM and a few FCA as well as a single eosinophilic adenoma, controls showed no other alterations.

Granulomata and other alterations of DM were rare in AFB_1- exposed medaka. This was in sharp contrast to control fish. Histopathologic examination of liver in moribund fish provided information on early responses to AFB_1 - induced toxicity. In Plate 4, figure B, an early change is presented. Small clusters of basophilic cells were seen. Higher magnification showed these cells to be biliary epithelial cells. They were easily contrasted with the appearance of adjacent rounded and glycogen laden hepatocytes (Plate 4, Figure B). In medaka sacrificed after three months of dietary exposure to AFB_1, small islands of basophilic cells alternated with eosinophilic cells (Plate 5, Figure A). This formed the toxicity pattern described above. FCA did not appear

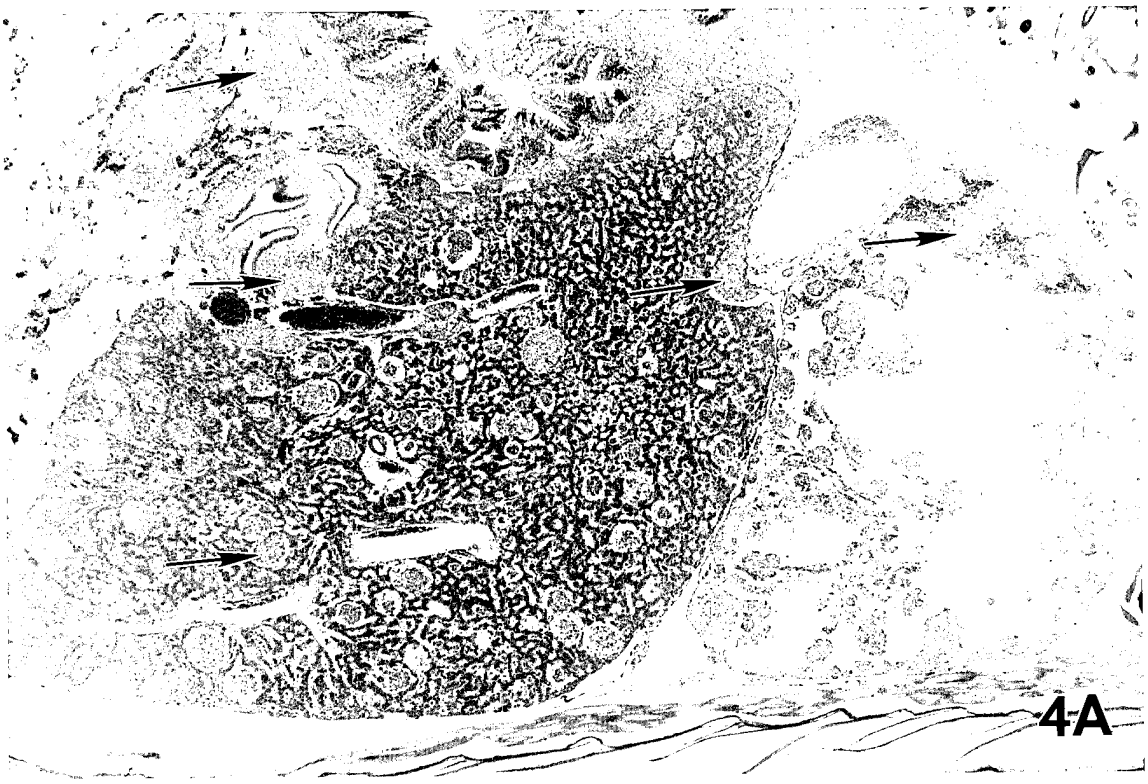
in a repeating pattern, were fewer in number and were comprised of hepatocytes. Often, two or more phenotypes of FCA were encountered in the same section (Plate 5, Figure B). In another section from the same fish, we found a hepatocellular carcinoma which contained a hepatocellular adenoma within its boundary (Plate 6, Figure A). At other times (Plate 6, Figure B), multiple tumors of hepatocellular and mixed hepatocholangiocellular components were seen within the same liver. All these multiple hits are consistent with chronic continuous exposure to the carcinogen which apparently served to initiate and promote. Three fish showed definitive evidence for metastasis (not shown in figures). Distant sites included kidney, skeletal muscle, and intestinal with adjacent mesentery.

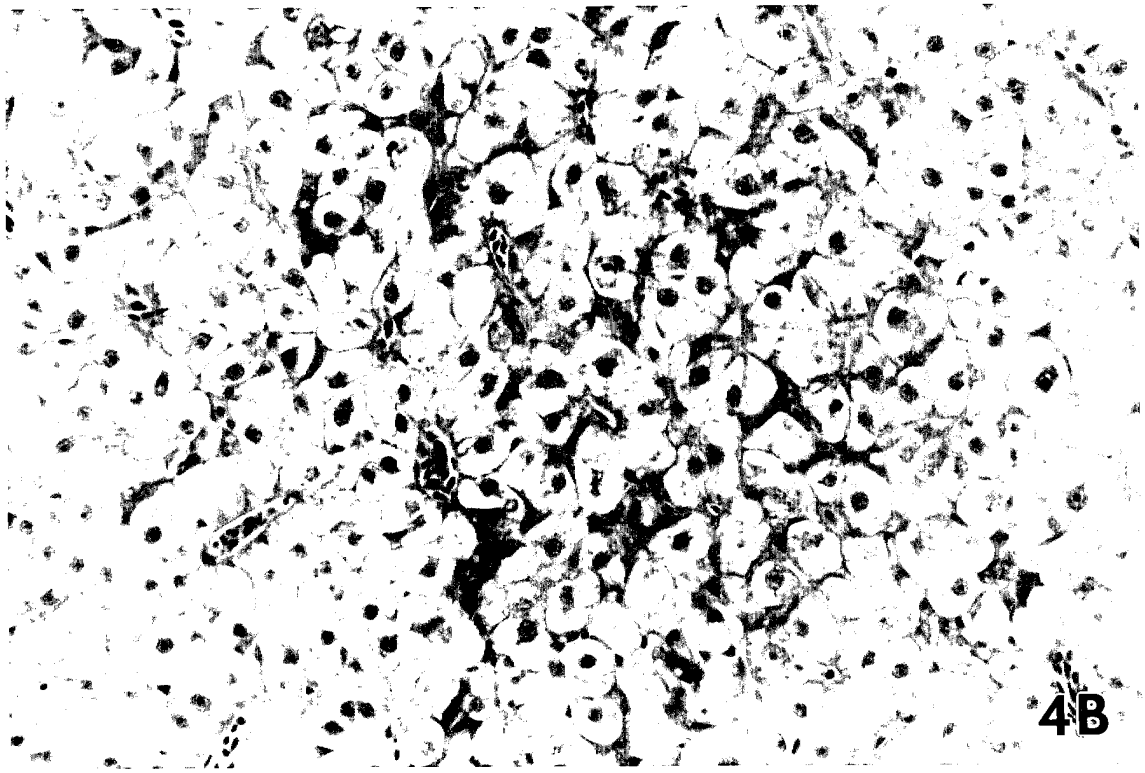
LEGENDS FOR FIGURES

Plate 4

FIGURE A. Disseminated mycobacteriosis in a female adult medaka fed control F/A-diet. Fish was necropsied at 7 months after the onset of the definitive AFB_1 study at 10 months of age. Multiple granulomata (arrows) are in liver, wall of gut, mesentery, heart, and attached to peritoneum of body wall. H&E X46.

FIGURE B. Liver section from moribund male medaka fed 3.00 ppm AFB_1 in PC-diet for one month. Basophilic cells contrast with adjacent hepatocytes. Abundance of biliary epithelial cells indicates proliferation of small bile ducts and ductules. H&E X460.


4A

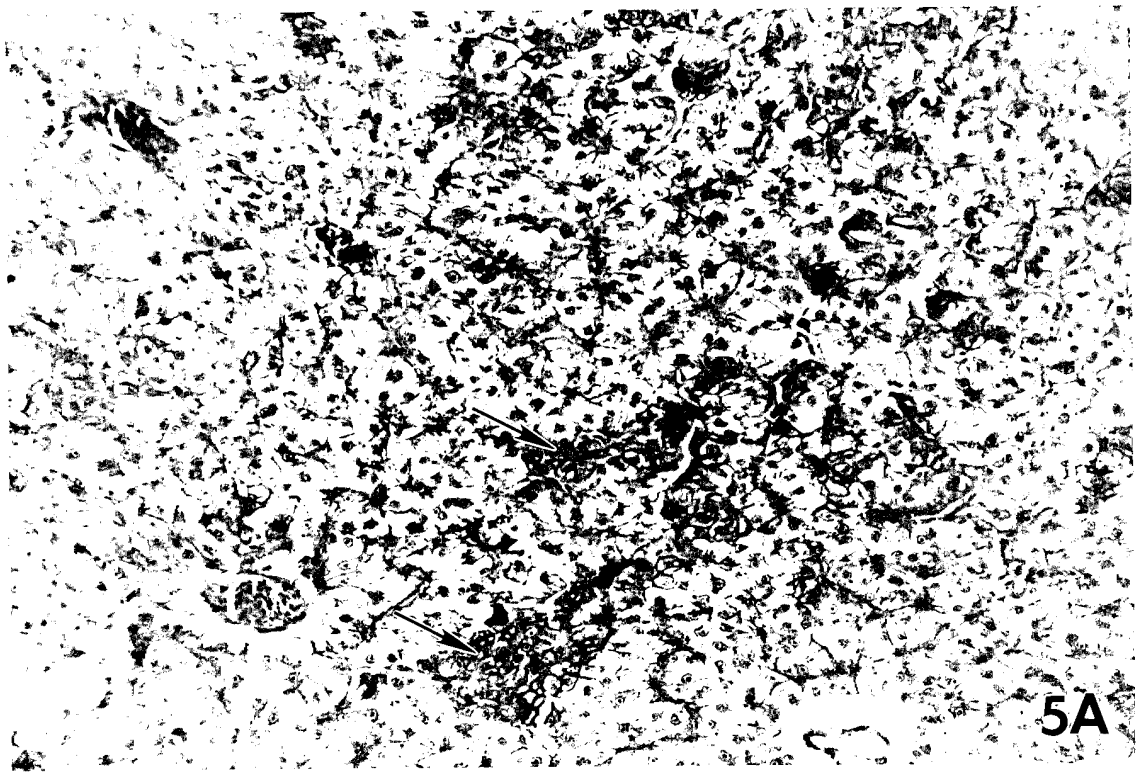

4B

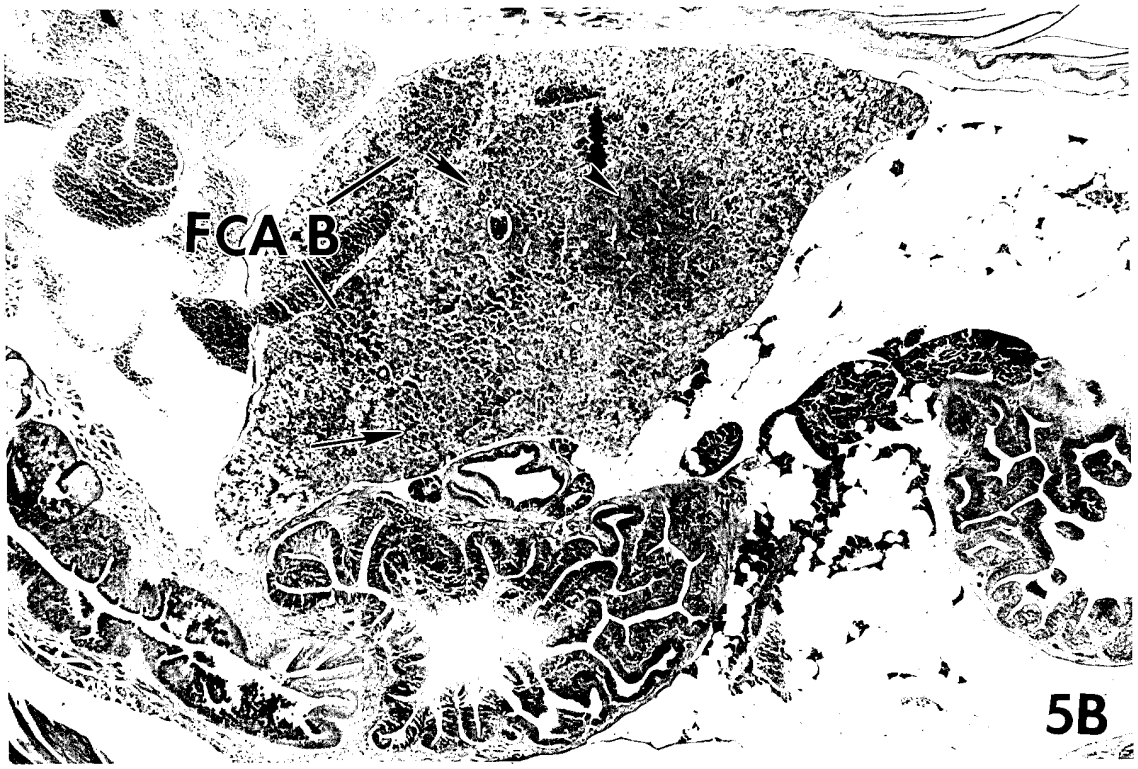
LEGENDS FOR FIGURES

Plate 5

FIGURE A. Section of liver from three month sampling. Fish was fed PC-diet containing 3.00 ppm AFB_1. Multiple focal lesions show basophilic cells (arrows) with characteristics intermediate between hepatocytes and biliary epithelial cells. We interpret these as regenerative islands and lesions were seen in carcinogen exposed fish. H&E X230.

FIGURE B. In another section of liver from the opposite side of the fish in Figure A, Plate 5 (above), multiple hits of the chronic AFB_1 exposure are shown. A hepatocellular adenoma (arrowheads) is found within a hepatocellular carcinoma (arrows). In addition two FCA with basophilic phenotype (FCA-B) are shown. H&E X46.


5A

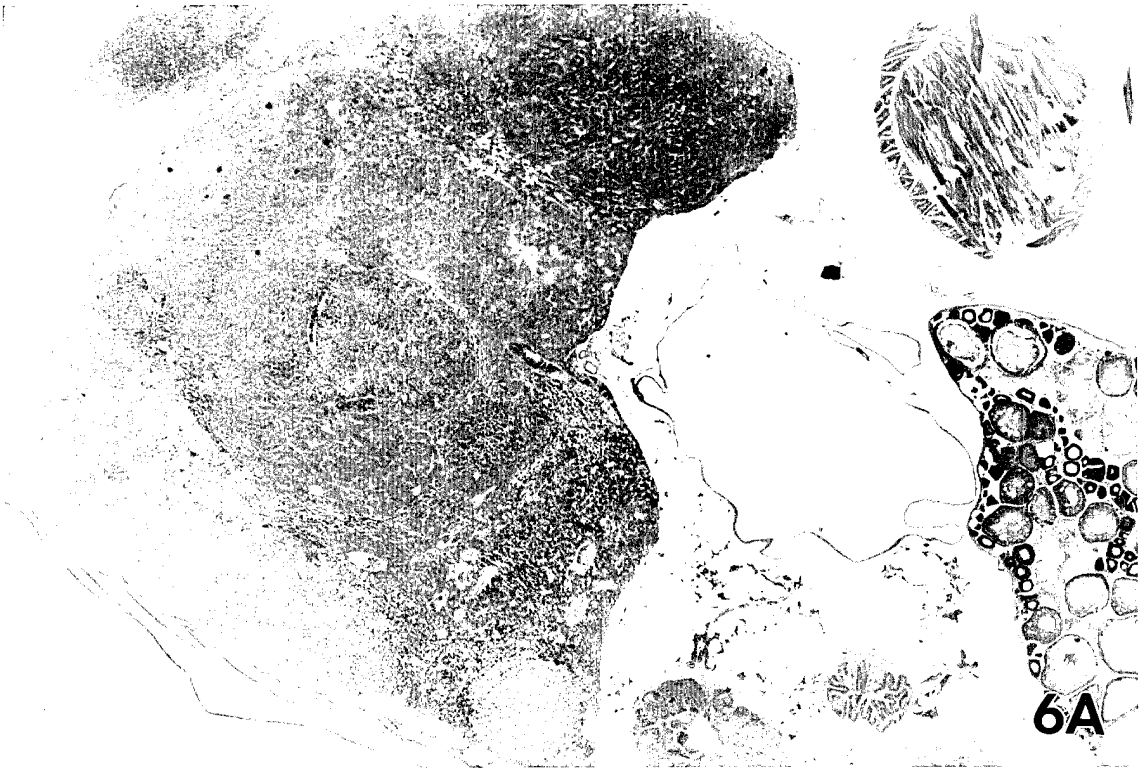

FCA-B
5B

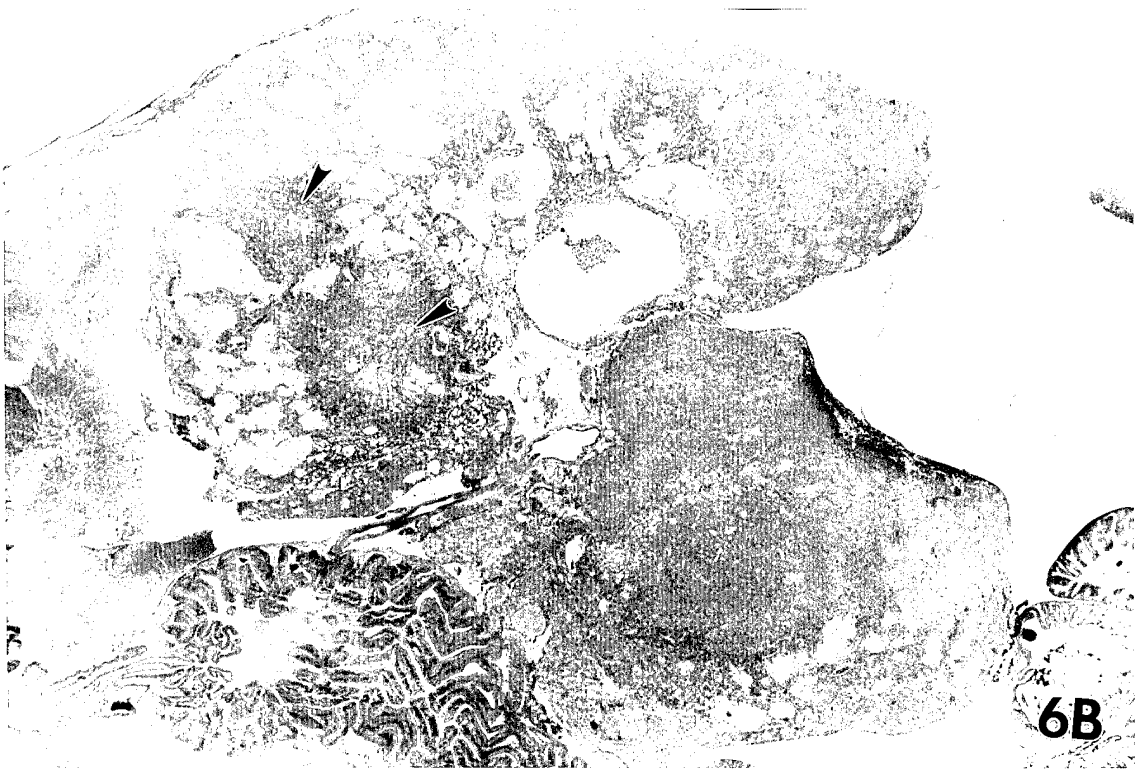
LEGENDS FOR FIGURES

Plate 6

FIGURE A. Female medaka from seven months sampling. Fish received PC-diet with 3.00 ppm AFB_1. This section shows a hepatocellular adenoma within a larger hepatocellular carcinoma. H&E X 46.

FIGURE B. In a male medaka sampled at seven months during the definitive study, a mixed hepatocholangiocellular carcinoma (arrowheads) was found within liver which also contained a hepatocellular carcinoma. Fish received F/A-diet containing 3.00 ppm AFB_1. H&E X 46.


6A


6B

DATA FOR GRAPHS (9-Mo samples):

			AFB1 Concentration (ppm)				
	Statistic	Diet	0	0.3	1.0	3	
	mean	**FA**	**.9**	**1.0**	**.59**	**.83**	
SH	±SE		.13	.25	.19	.54	
	N		8	16	8	8	
	freq.			.44	0	.25	
	mean	**PC**		**.8**		**.63**	
	±SE			.23		.42	
	N		74	26	32	6	
	mean	**FA**	**1.4**	**2.0**	**2.0**	**1.7**	
HMA	±SE		.09	.14	.17	.21	
	N		8	16	8	8	
	mean	**PC**	**1.5**	**1.9**	**1.6**	**1.8**	
	±SE		.33	.14	.18	.25	
FCA	N	FA	73	26	32	6	
	freq.		.25	.7	.63	.33	
	N	PC	9	16	8	8	
	freq.		.22	.4	.88	.88	
NEO	N	FA	73	26	31	6	
	freq.		.01	.2	.26	.33	
	N	PC	9	16	8	8	
	freq.		0	.1	0	.25	
							Totals
SEX	M	FA	34	13	15	6	68
	F		38	9	9	0	56
	NP		3	5	8	0	16
	M	PC	2	6	2	6	16
	F		7	9	1	2	19
	NP		0	1	5	0	6

Discussion

Results of this study have shown that the PC-diet may be used in conjunction with AFB_1 in a long-term dietary exposure. When compared against the conventional regime, the definitive study showed survival in both control groups to be indistinguishable by statistical methods. The finding of disseminated mycobacteriosis, an unexpected result of the study, appears in medaka at or slightly before 9 months of age. Our housing conditions of nonstop 16-hr light and 8-hr darkness at elevated temperature (25°C) may add to the stress encountered by these fish. With added age the tendency of medaka to develop mycobacteriosis overcame host inherent protective mechanisms. From the mycobacterial culture studies performed, the organism is present but probably in smaller numbers and, if fish are not stressed, the problem is not made manifest. It was interesting to note (histogram figure 3-17) that the control groups for both diets had higher severity scores for disseminated mycobacteriosis than did their corresponding treatment groups. For each diet, increasing the concentration of carcinogen appeared to diminish the disseminated mycobacteriosis severity score. This should not be regarded as definitive information since the severity scores are relative. However, the same criteria were applied for all tissues in determining the various scores. Another form of degenerative condition, spongiosis hepatis, was encountered in medaka fed the F/A-diet. The severity scores and incidence data indicated that this condition was more likely to be associated with feeding on the FA than the PC-diet. Control F/A-diet fed fish showed spongiosis hepatis while control PC-fed fish did not. Since PC-diet fed fish exposed to AFB_1 showed spongiosis hepatis, and both controls and experimental fish of the F/A-diet groups showed the condition, we are assuming that there is some factor present in the F/A-diet that is associated with the formation of this lesion.

Atrial phagocyte hypertrophy (APH) was a condition that was found with greater severity score in all PC groups versus their corresponding FA fed cohorts. APH is part of a spectrum of changes seen in disseminated mycobacteriosis. APH may represent an early stage of the disease. The toxicity effects of AFB_1 were apparent when fish of the pilot study were removed from exposure to the carcinogen and placed on control diet. One indicator of return toward control morphology was the presence of greater areas of the cytoplasm occupied by glycogen. This information, shown in Figure 3-20, indicates that some of the stress placed on medaka of the study was removed by their removal from AFB_1 exposure. Active exposure to AFB_1 was associated with glycogen depletion.

The mosaic pattern of alternating basophilic and eosinophilic regions within liver of AFB_1-exposed fish may represent a biomarker for exposure to this compound. While rare control fish also showed this lesion, they were very infrequent and not as extensive as the conditions encountered in the AFB_1-exposed fish. When the AFB_1-exposed fish were removed from carcinogen exposure, disappearance of the mosaic pattern was noted.

The holding and chronic exposure of fish under static renewal conditions proved unsatisfactory. The pilot study was characterized by high mortality and extensive disseminated mycobacteriosis. The reduced mortality in the definitive study that used a recirculating system indicated that much of the husbandry problems of the static exposures had been overcome by use of the recirculating system. Mortality data in the 1992 pilot study and the 1994 definitive study were markedly different (Fig. 3-27). The control mortality for the 1994 study was between 20 and 30% and was similar in both diets. The 1994 mortality for the carcinogen-exposed fish showed that the PC-fed fish had a mortality of about 40% while the FA-fed fish mortality was

greater (60%). We suspect that the FA-fed fish are progressing to tumor formation more rapidly than are PC-fed fish and that this accelerated development of the AFB_1-induced hepatic neoplasms is contributing to this mortality. The improved survival of PC-fed fish in some of the aquaria of the 1992 pilot study may be due to earlier mortality and capacity for surviving fish to grow due to less competition.

The statistical testing for survival in the definitive AFB_1 study indicated that the two control groups were not different. Confirmation of increased mortality for the FA-fed fish exposed to AFB_1 was obtained. This group was significantly different from all other groups. It is clear that addition of 3.0 ppm AFB_1 to F/A-diet significantly decreased survival of individuals. With the PC-fed groups, the percent medaka surviving was different but not strongly different from that of the AFB_1 group.

The results of the pilot and definitive AFB_1 studies as well as that of the DEN studies (Chapter 2) indicate that fish given the F/A-diet with two days of supplementation by brine shrimp progress to tumor formation more rapidly than do fish fed only the PC-diet. The comparison of the two diets is interesting. First, the F/A-diet with two days supplementation may be the best regime for more rapid production of tumors. However, it has a drawback of being a commercially available ration, the formulation of which may be altered and the composition of which is not exactly known. While the PC-diet may lack nutritional factors which positively modulated FA-fed fish, the composition of the diet is known and the diet can be made with resulting consistency between batches. PC-diet resulted in sufficient frequency for bioassay. It is interesting to compare the two diets and their relative rates for FCA development and hepatic neoplasm development. This gives us not only a picture of the properties of the two dietary

regimes but a better handle on understanding progression from normal liver to overt neoplasms. In the pilot and definitive studies, the frequency of FCA diminished in the FA-fed group as their tumors developed. This suggests that at least some of the FCA are incorporated in the eventual neoplasms. Also, the continuous feeding of the diet for a period of six months led to conditions that were more difficult to interpret within lesions. Whereas the DEN initiation scheme for hepatic neoplasms (see Chapter 2) was one which produced a high yield of tumors. The lesions were comprised of uniform phenotypes. A consistent finding within the AFB_1-exposed fish over the six months duration was the finding of "lesions within lesions." These are adequately illustrated in Plates 4 through 6. This indicates that some cells are being initiated while other clones have already achieved the status of FCA and/or tumors.

With both diets, female fish of the individual groups appear to develop foci and neoplasms more rapidly than their male cohorts. Gender specific differences have been seen in other laboratory models such as the rainbow trout (*Oncorhynchus mykiss*). It is interesting to speculate that the production of eggs by females under conditions of the test may have been associated with endocrine related trophic factors on liver which promoted the development of the FCA and neoplasms. This subject is being addressed in our laboratory currently under funding from alternative sources.

In conclusion, the PC-diet proved satisfactory under conditions of long-term dietary exposure during carcinogen bioassay. Differential rates in tumor production were encountered and the purified diet took longer for tumors to develop. This may indicate that positive modulatory factors are present in the commercially available ration and/or the brine shrimp. Static renewal systems proved unsatisfactory for culture of fish over the six-month exposure duration. Improved

culture and survivability was obtained by use of a flow-through partially recirculating system. Regardless of the diet fed, chronic exposure to AFB_1 in the medaka produces a toxicity state evidenced by panhepatic mosaic pattern development of alternating basophilic and eosinophilic cells. This condition disappears upon removal of the fish from exposure. The long-term feeding of medaka extended earlier observations by Hatanaka et al. (1982) and more fully documents the progression from normal liver to overt neoplasms. Mycobacteriosis is a problem of moderate to severe magnitude that needs to be addressed in the medaka model. The exposure of medaka to AFB_1 reduced histologic evidence for mycobacteriosis and appeared to have a protective effect against this condition. Gender specific differences in rate of tumor development were encountered with females being more susceptible. A quantitative relationship between level of AFB_1 in the diet and number of AFB_1-induced DEN adducts was established. In addition, the number of DNA adducts were quantitatively and positively related to frequency of tumor formation. This is the first production of dose response characteristics for the medaka tumor model.

7. CONCLUSIONS

The overall goal of the research funded by this contract is to: develop a sensitive fish model for studying carcinogenic potential and modulatory effects of environmental agents on carcinogenesis.

To reach this overall goal, four enabling objectives were identified.

1) Produce a nutritionally adequate, open-formula purified diet for medaka. The completed research has met this objective.

The overall nutritional adequacy of a purified casein-based diet (PC-diet) for the medaka (*Oryzias latipes*) was evaluated and compared with three diets: commerically available flaked fish food (FL-diet), live newly hatched *Artemia* (A-diet), and a combination of FL-diet plus A-diet (F/A-diet). Survival, growth, reproductive success, general and liver histopathology, and selected hepatic enzyme activities were compared in medaka from first feeding through reproductive maturity. The PC-diet proved adequate in all of the above criteria. When compared with fish fed F/A-diet, an initial lag in early growth rates (i.e., 0 to 30 days) occurred with the fish fed PC-diet. The FL-diet alone was not nutritionally adequate for medaka, resulting in poor growth, reduced reproductive success, lower survival, and emaciation. A significant number of spinal deformities (5.4%) were noted in medaka fed the F/A diet. Ethoxycoumarin O-deethlyase and glutathione S-transferase activities were monitored and a trend toward increasing activity with age was noted. This suggests that PC- and F/A-diets provide adequate nutrition for development of the xenobiotic metabolizing enzymes necessary for detoxification and activation of endogenous and foreign compounds. The PC-diet supported good survival, growth, reproduction and normal histology. This diet provides a standardized, nutritionally adequate, and consistent alternative to undefined conventional diets and is less likely to contain the range of xenobiotics possible in whole, live food.

2) Determine levels of xenobiotic metabolizing enzymes in medaka fed this and a conventional diet;

This objective was met and is described in Chapter 1 (above).

3) Establish incidences of liver tumors in medaka fed the open-formula diet; and

4) Compare the frequency of liver tumor formation in carcinogen-initiated medaka fed the

open formula diet versus those fed a conventional dietary regime.

Objectives 3 and 4 were done together and are described in the work of Chapters 2 and 3 (Body, this report).

A recently developed purified casein-based diet (PC-diet) for maintenance of medaka (*Oryzias latipes*) was used in comparison studies with a commonly employed commercial flake diet to determine suitability of the purified ration for use in carcinogenicity studies. From day one after hatch, fish were fed either the PC-diet or a commercial flake ration supplemented with two days of *Artemia sp.* each week (F/A diet). At 21 days of age, both dietary groups of fish were exposed separately to aqueous solutions of 350 ppm (nominal) of diethylnitrosamine (DEN) for 48 hr. This regimen has been shown to yield tumors of the liver after a period of latency usually extending for six to nine months. The use of this design afforded an opportunity to compare two dietary regimes over a period of time that would include initiation, promotion and progression of hepatic neoplasms. Foci of cellular alteration were first seen after month one. After month three, initial neoplasms were encountered and hepatic tumors were found in animals fed each diet. At 259 days after initiation of exposure, and after one of the groups had shown a 50% incidence of tumors, all remaining fish were sampled and their livers processed for histopathology. Fish fed the F/A diet had a higher incidence of hepatocellular foci (58 basophilic, 54 eosinophilic and 23 clear cell) than did fish fed the PC-diet (30, 39 and 15 respectively). Also, incidence of hepatocellular neoplasms was higher (73 versus 47) in the former than the latter. The PC-diet yielded sufficient tumors for statistical analysis and, given its interbatch consistency, is recommended for further use. Growth of DEN-exposed fish was similar regardless of the diet fed and depression of growth over the course of the study indicated

that DEN, and not the diets, was the major factor responsible in weight differences.

Studies with AFB_1 were completed and led to quantitation of AFB_1-DNA adducts as a function of AFB_1 in the diet. With increasing levels of AFB_1, increasing adduct formation occurred. This dose response effect also characterized the tumor frequency. This finding firmly establishes the medaka liver tumor model from the standpoint of dosimetry, genotoxicity and tumor response. Female medaka proved more sensitive to AFB_1 by reaching FCA and neoplastic endpoints more rapidly. This occurred in both diets. PC-diet (purified open-formula ration) proved adequate as an exposure medium over a 6-month dietary study. Fish fed the F/A diet and exposed to AFB_1 (3.0 ppm) reached tumor more rapidly and in greater frequency than did PC-fed fish. This suggests positive tumor modulatory factors are present in the F/A versus the PC-diet.

A serious problem was encountered in fish fed either diet when held under bioassay conditions for periods in excess of six months. This was the occurrence of granulomata within skeletal muscle, mesentery, liver, kidney, heart, and other viscera. The condition, termed disseminated mycobacteriosis, apparently develops from organisms that are normally found within aquatic systems. Future work is highly recommended in order to determine if specific strains of medaka are more resistant to mycobacteriosis and to determine culture conditions that may enable fish to better withstand this ubiquitous organism. Culture and husbandry conditions might include investigation of the need to maintain fish at elevated temperatures, investigation of alternation of lighting cycles for fish under bioassay, investigation of flow-through single pass culture versus recirculating systems, and the intensity and residency time needed for UV irradiation to promote killing of organisms. In addition, the formulation of binders for the purified ration may result in less dissolution into water with less production of microorganisms

and better nutrition for fish. The use of vitamin mixes that are not water soluble may further improve the nutritional quality of the purified ration. In addition, the use of purified fatty acids may bypass all need for oils from fish or agricultural sources. It is now known that levels of compounds such as polychlorinated biphenyls and some organochlorines are present in all corn oils and likely would be found in other vegetable oils as well. Since medaka eat relatively small amounts of diet in their culture versus that of larger fish, it may still be cost effective to prepare totally synthetic and defined ration.

Acknowledgements

In addition to the research group, Ms. L. Parker, Ms. S. Lester, Mr. M. Anderson, S.J. Teh, M.S., Dr. M. Okihiro, Dr. D. Conklin, Dr. G. Marty and Ms. D. DeKoven, the author gratefully acknowledges the assistance of Dr. Chris Calvert, Department of Animal Science, UC Davis. I also thank Michael Donalson, Thom Strella, and Nancy Baum, for their expert care and maintenance of the fish.

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APPENDIX I

1- (C#) Case Number
2- (S#) Slide Number

File = C:\SC\DATA\ALASKA\AFB1_92A.CAL
Processing: fish were cassetted, fixed in Boiun's, then transected midsagitally and transferred to 70% EtOH at 10 am on 3-4-93 (i.e., about 36 h in Bouin's)

4- (DOD) Date of death; ENTER "DAT(mm,dd,yy)"
5- (FIX) Fixation, SF=sample fixed, MF=moribund, DF=dead (DFF = fresh), AF= autolyzed
6- (DOH) Date of hatching; enter "DAT(mm,dd,yy)"
7- Age (days)
8- Sex, M= male, F= female, U= unknown, H= hermaphrodite.
9- (HG) Hepatic glycogen/vacuolation 1=min. 2=mod. 3= abund. NP=not present, A= autolyzed
10- (VE) Vacuolar encephalopathy 0=none 1=min 2=mild 3=mod 4=severe, NP , A
11- (A) Autolysis; 1=min, 2=mild, 3=mod, 4=sev
12- Lesions (LES); 0=no histologic lesions; 1=lesions present

STANDARD LESION SCORES: 1 = minimum (min), 2 = mild, 3 = moderate (mod), 4 = severe (sev)

13- (DM) Disseminated Mycobacteriosis;
old abbreviation = BIDD (basophilic inclusion disease, disseminated)
MADD (mycobacterium associated disease, disseminated)
14- (GPC) Granulomatous pericholangitis;
old abbreviation = MS (malignant spongiosis)
15- (OG) Granulomatous oophoritis;
16- (APH) Atrial phagocyte hypertrophy;
17- (SH or HS) Spongiosis hepatis;
18- (GGH) Gas gland hyperplasia;
19- (MDN) Myofiber degeneration and/or necrosis;
20- (HN) Hepatic necrosis; individual cell or zonal;
21- (ENT) enteritis;
22- (HMA) Hepatic macrophage aggregates;
23- (RMA) renal macrophage aggregates;
24- (FLK) Flukes or monogenetic trematodes;
25- (GLH) gill lamellar epithelial hyperplasia
26- GLT (gill lamellar telangiectasis)
27- HFC (hepatic fatty change)
28 - (MBE) mixed basophilic and eosinophilic cells in liver
29- (FCA) foci of cellular alteration, liver; P = present; see "comments" for type
30- (NEO) Neoplasia; P = present; see "COMMENTS" for type.
31- (XXX) other lesions; see under "comment"
FCH (fibrocartilaginous hyperplasia, mandible)
RTN (renal tubular necrosis)

			Exposure				Weight																							
#	C#	S#	Diet	Conc.	FIX	AGE	(g)	SE	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	FCA	NEO	MBE	S#
1	93H36-	9	FA	0	SF	366	0.5309	F	1	2	2	1	0		3	1	1	2	1			0	2					1	0	0	9
2	93H36-	14	FA	0	SF	366	0.3945	F	1	1	2	1	0		2	1	0					0	2					0	0	0	14
3	93H36-	25	FA	0	SF	366	0.3082	F	1	0	2	1	0		2	3	0					0	2					0	0	0	25
4	93H36-	33	FA	0	SF	366	0.391	U	1	2	2	1	3			2	0					1	2					1	0	0	33
5	93H36-	53	FA	0	SF	366	0.2639	F	1	0	2	1	0			1	0					0	2					0	0	0	53
6	93H36-	71	FA	0	SF	366	0.2468	M	1	0	3	1	3			2	0		2			1	1					0	0	0	71
7	93H36-	77	FA	0	SF	366	0.4938	M	1	0	2	1	1			0	0			1		2	2					0	0	0	77
8	93H36-	78	FA	0	SF	366	0.4419	M	1	2	3	1	2			2	0					2	2					0	0	0	78
9	93H36-	85	FA	0	SF	366	0.3416	M	1	1	3	1	0			1	1					0	1					0	0	0	85
10	93H36-	91	FA	0	SF	366	0.339	F	1	1	2	1	0		2	2	2					2	2					0	0	0	91
11	93H36-	92	FA	0	SF	366	0.2986	F	1	1	3	1	0		2	1	0	2				1	1					0	0	0	92
12	93H36-	107	FA	0	SF	366	0.4418	F	1	0	3	1	2		2	2	0					1	2					0	0	0	107
13	93H36-	108	FA	0	SF	366	0.3269	M	1	4	2	0	0			1	0					1	2					0	0	0	108
14	93H36-	110	FA	0	SF	366	0.3668	F	1	0	2	1	2		1	2	0					1	2					0	0	0	110
15	93H36-	114	FA	0	SF	366	0.275	M	1	1	3	0	0			1	0					1	1					0	0	0	114
16	93H36-	123	FA	0	SF	366	0.372	F	1	2	3	1	0		1	3	2					1	3					0	0	0	123
17	93H36-	124	FA	0	SF	366	0.3086	M	1	1	2	1	0			2	2					0	2					1	0	0	124
18	93H36-	135	FA	0	SF	366	0.3104	F	1	1	2	1	0		2	2	3					0	2					0	0	0	135
19	93H36-	139	FA	0	SF	366	0.186	F	1	2	2	1	0		2	0	0					1	2		1			0	0	0	139
20	93H36-	160	FA	0	SF	366	0.44	M	1	1	2	0	0			0	0					0	2					0	0	0	160

APPENDIX I
AFB1-92: 6-MONTH SAMPLES

#	C#	S#	Exposure Diet	Exposure Conc.	FIX	AGE	Weight (g)	SE	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	FCA	NEO	MBE	S#
21	93H36-	13	PC	0	SF	366	0.3976	M	1	2	3	1	1			2	0					0	2					0	0	0	13
22	93H36-	20	PC	0	SF	366	0.3605	M	1	3	2	1	1			3	0		2			1	0					0	0	0	20
23	93H36-	30	PC	0	SF	366	0.4471	F	3	2	2	1	0		2	3	0					0	1					0	0	0	30
24	93H36-	43	PC	0	SF	366	0.2568	F	1	2	1	1	0		2	2	0					2	2					0	0	0	43
25	93H36-	44	PC	0	SF	366	0.3324	M	1	0	1	0	0			0	0					0	1					0	0	0	44
26	93H36-	60	PC	0	SF	366	0.2379	F	1	1	3	1	0		2	2	0	2	1			1	1					0	0	0	60
27	93H36-	63	PC	0	SF	366	0.64	M	2	1	3	1	0			2	0	3				0	2					1	0	0	63
28	93H36-	66	PC	0	SF	366	0.3907	M	1	1	3	0	0			1	0					0	2					0	0	0	66
29	93H36-	74	PC	0	SF	366	0.7031	F	2	1	2	1	2		1	NP	0		2			1	2					1	0	0	74
30	93H36-	80	PC	0	SF	366	0.8543	F	2	2	2	1	2			2	2					1	2					0	0	0	80
31	93H36-	96	PC	0	SF	366	0.4522	M	2	1	3	1	0			2	0	2				0	1					0	0	0	96
32	93H36-	101	PC	0	SF	366	0.2956	M	1	2	1	1	2			1	0					0	2					0	0	0	101
33	93H36-	106	PC	0	SF	366	0.3746	F	1	1	3	1	3		2	2	0		2			1	1					0	0	0	106
34	93H36-	117	PC	0	SF	366	0.2541	M	1	2	1	1	0			2	0	2	2			1	2					0	0	0	117
35	93H36-	129	PC	0	SF	366	0.6116	F	2	1	2	1	3		2	3	0		3			0	0					0	0	0	129
36	93H36-	143	PC	0	SF	366	0.1702	F	1	1	2	1	0		2	3	0		3			2	3					0	0	0	143
37	93H36-	155	PC	0	SF	366	0.3145	M	1	0	2	1	0			2	0		1			1	1					0	0	0	155
38	93H36-	158	PC	0	SF	366	0.5881	F	1	2	2	1	0		2	2	0		2			2	2		2	1		0	0	0	158
39	93H36-	161	PC	0	SF	366	0.7707	F	2	2	2	1	2		1	3	0					3	2					0	0	0	161
40	93H36-	164	PC	0	SF	366	0.457	F	1	1	3	1	0		2	2	0					2	2		2			0	0	0	164
41	93H36-	31	FA	0.3	SF	366	0.1984	F	1	0	2	1	0		1	2	2					3	3					0	0	0	31
42	93H36-	34	FA	0.3	SF	366	0.3133	M	1	2	3	1	0			2	0					2	2					1	0	0	34
43	93H36-	40	FA	0.3	SF	366	0.2735	M	2	0	2	1	0			2	0					1	1					0	0	0	40
44	93H36-	42	FA	0.3	SF	366	0.3528	F	3	2	3	1	0		2	1	2		1			2	2					1	0	0	42
45	93H36-	45	FA	0.3	SF	366	0.621	F	2	0	3	1	1		2	2	0					1	2					1	0	0	45
46	93H36-	49	FA	0.3	SF	366	0.3863	F	3	1	1	1	0		2	0	0					1	2					1	0	0	49
47	93H36-	57	FA	0.3	SF	366	0.6104	M	2	1	3	1	0			0	2					2	1					1	0	0	57
48	93H36-	64	FA	0.3	SF	366	0.425	M	2	0	1	1	2			2	0					1	1					0	0	0	64
49	93H36-	75	FA	0.3	SF	366	0.346	F	2	1	2	1	0		2	0	2					2	2					1	1	0	75
50	93H36-	82	FA	0.3	SF	366	0.4108	F	2	2	2	1	1		2	1	2			3		2	2					1	0	0	82
51	93H36-	86	FA	0.3	SF	366	0.3498	F	1	1	2	1	0		2	2	0		1			0	3			2		1	0	0	86
52	93H36-	89	FA	0.3	SF	366	0.388	M	2	1	2	1	0			1	0					1	2					0	0	0	89
53	93H36-	97	FA	0.3	SF	366	0.3342	M	1	0	2	1	0			2	0					2	2					0	0	0	97
54	93H36-	119	FA	0.3	SF	366	0.447	M	3	2	3	1	0			2	0		1			1	2					0	0	0	119
55	93H36-	134	FA	0.3	SF	366	0.3684	F	2	1	2	1	0		1	2	0			1		3	2					1	0	1	134
56	93H36-	137	FA	0.3	SF	366	0.3447	M	2	0	3	1	0			1	0	2				2	2				1	1	0	1	137
57	93H36-	145	FA	0.3	SF	366	0.2927	M	2	3	3	1	0			2	2					3	2					0	0	0	145
58	93H36-	148	FA	0.3	SF	366	0.4067	M	1	1	3	1	0			2	2					2	2		3			0	0	0	148
59	93H36-	162	FA	0.3	SF	366	0.2989	F	1	2	2	1	0		2	1	0					3	2					1	0	1	162
60	93H36-	163	FA	0.3	SF	366	0.4217	F	3	1	2	1	0		2	1	2					2	2					0	0	0	163
61	93H36-	11	PC	0.3	SF	366	0.4905	M	1	3	2	1	3			3	1		1			1	0					0	0	0	11
62	93H36-	36	PC	0.3	SF	366	0.635	F	2	2	2	1	2		2	2	0					3	2					0	0	0	36
63	93H36-	46	PC	0.3	SF	366	0.5562	M	1	3	3	1	2			NP	0		1			1	1					0	0	0	46
64	93H36-	56	PC	0.3	SF	366	0.5411	F	1	1	3	1	0		2	2	0					1	2					1	0	0	56
65	93H36-	58	PC	0.3	SF	366	0.459	M	1	2	3	1	0			2	0					1	2					1	0	0	58
66	93H36-	65	PC	0.3	SF	366	0.6781	F	1	0	2	1	0		1	2	0					0	2					0	0	0	65
67	93H36-	69	PC	0.3	SF	366	0.58	M	1	3	3	1	0			2	1					2	2					0	0	0	69
68	93H36-	84	PC	0.3	SF	366	0.4869	M	1	1	2	1	0			1	0	4				0	2					0	1	0	84
69	93H36-	87	PC	0.3	SF	366	0.647	F	1	0	3	1	0		2	2	1			1		2	2					1	0	0	87
70	93H36-	90	PC	0.3	SF	366	0.4826	F	1	1	2	1	0		2	3	0	2				2	1					0	0	0	90
71	93H36-	98	PC	0.3	SF	366	0.2902	M	1	2	2	1	0			2	0				2	2	2					0	0	0	98

#	C#	S#	Exposure Diet	Exposure Conc.	FIX	AGE	Weight (g)	SE	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	FCA	NEO	MBE	S#
72	93H36-	100	PC	0.3	SF	366	0.3968	M	1	1	2	1	?			3	0					3	2					0	0	0	100
73	93H36-	113	PC	0.3	SF	366	0.3882	M	1	1	2	1	0			2	0					2	1					0	0	0	113
74	93H36-	120	PC	0.3	SF	366	0.4248	M	1	2	3	1	0			2	0					1	2					0	0	0	120
75	93H36-	125	PC	0.3	SF	366	0.6089	F	1	3	3	1	0		1	2	0		2	2		3	2				2	1	0	1	125
76	93H36-	127	PC	0.3	SF	366	0.44	F	1	0	1	1	2		3	3	0		3			2	2		4			0	0	0	127
77	93H36-	128	PC	0.3	SF	366	0.4684	M	1	1	2	1	3			2	0		1			2	2					0	0	0	128
78	93H36-	132	PC	0.3	SF	366	0.3211	M	1	0	2	1	0			2	0		2			0	2					0	0	0	132
79	93H36-	141	PC	0.3	SF	366	0.486	M	1	3	1	1	2			3	0		1			1	2					0	0	0	141
80	93H36-	159	PC	0.3	SF	366	0.5263	F	2	4	2	1	0		2	2	0					1	1					0	0	0	159
81	93H36-	4	FA	1	SF	366	0.4426	F	2	1	2	1	0	3		0	0	2				1	2			2		1	0	0	4
82	93H36-	10	FA	1	SF	366	0.183	F	2	1	2	1	0		2	1	1					2	2					1	0	1	10
83	93H36-	12	FA	1	SF	366	0.3295	M	2	2	3	1	2			2	1					2	2					1	0	1	12
84	93H36-	15	FA	1	SF	366	0.2896	F	3	0	3	1	0		1	1	0					1	2					1	0	0	15
85	93H36-	23	FA	1	SF	366	0.4121	M	2	0	2	1	0			0	0	2				1	2					1	0	1	23
86	93H36-	29	FA	1	SF	366	0.4842	U	1	2	2	1	0			2	0					1	1					0	0	1	29
87	93H36-	38	FA	1	SF	366	0.2879	M	2	0	1	1	0			1	0					1	2					1	0	1	38
88	93H36-	47	FA	1	SF	366	0.3218	M	2	1	3	1	0			2	0					0	2					1	0	0	47
89	93H36-	62	FA	1	SF	366	0.3034	M	1	2	2	1	0			2	0					2	2					0	0	0	62
90	93H36-	73	FA	1	SF	366	0.4245	M	1	0	2	1	0			2	1	2				2	2					1	0	1	73
91	93H36-	81	FA	1	SF	366	0.3555	F	2	2	2	1	0		2	2	0					2	2					0	0	0	81
92	93H36-	95	FA	1	SF	366	0.9101	M	2	2	2	1	2			2	0					1	1					0	0	1	95
93	93H36-	99	FA	1	SF	366	0.3707	M	1	2	2	1	1			1	2					2	2					0	0	0	99
94	93H36-	109	FA	1	SF	366	0.3955	M	3	2	1	1	0			1	2	2				2	2					0	0	1	109
95	93H36-	116	FA	1	SF	366	0.3411	M	1	2	3	1	3			2	0					2	1			2		0	0	0	116
96	93H36-	121	FA	1	SF	366	0.4021	M	2	2	1	1	2			3	0			2		2	2					0	0	1	121
97	93H36-	131	FA	1	SF	366	0.2535	F	1	2	3	1	0		1	2	0			2		2	2		2			1	0	1	131
98	93H36-	156	FA	1	SF	366	0.6581	F	1	0	2	1	0		2	2	1			1		3	2					1	0	1	156
99	93H36-	157	FA	1	SF	366	0.3392	M	1	1	2	1	0			1	3					1	3					0	0	1	157
100	93H36-	165	FA	1	SF	366	0.5742	F	2	1	3	1	0		2	2	3			2		1	2		2			1	1	0	165
101	93H36-	7	PC	1	SF	366	0.2976	F	1	1	2	1	0		2	2	0		2			3	2					1	1	1	7
102	93H36-	17	PC	1	SF	366	0.2289	U	1	2	1	1	0			1	2					2	2					0	0	0	17
103	93H36-	19	PC	1	SF	366	0.4564	M	1	1	2	1	2			2	0					2	2					0	0	0	19
104	93H36-	32	PC	1	SF	366	0.5445	M	1	4	2	1	1			2	0		2			2	2				2	1	0	0	32
105	93H36-	41	PC	1	SF	366	0.588	M	1	0	3	1	2			3	2					1	1					1	0	0	41
106	93H36-	59	PC	1	SF	366	0.472	F	1	0	2	1	0		1	3	0					1	1				1	1	0	1	59
107	93H36-	79	PC	1	SF	366	0.2998	F	1	1	3	1	0			1	0		2			2	2					0	0	0	79
108	93H36-	88	PC	1	SF	366	0.2902	M	2	2	2	1	2			3	0			3		2	2				1	0	0	0	88
109	93H36-	94	PC	1	SF	366	0.3101	M	2	2	2	1	0			2	0					2	2					0	0	0	94
110	93H36-	102	PC	1	SF	366	0.56	F	2	0	2	1	0		1	3	2		3			2	2					1	1	0	102
111	93H36-	104	PC	1	SF	366	0.499	M	1	3	3	1	0			2	0					2	2					1	0	1	104
112	93H36-	122	PC	1	SF	366	0.5031	M	1	2	2	1	0			2	0		3			1	1		1			1	0	0	122
113	93H36-	130	PC	1	SF	366	0.457	F	3	1	2	1	0		2	2	2					2	2		3			1	0	1	130
114	93H36-	136	PC	1	SF	366	0.473	M	1	2	2	1	2			2	0		3			2	1		2			1	0	0	136
115	93H36-	138	PC	1	SF	366	0.4365	M	1	1	2	1	0			2	0		3			2	2					0	0	0	138
116	93H36-	144	PC	1	SF	366	0.4533	F	3	1	3	1	0	4	2	3	3					3	2					1	0	0	144
117	93H36-	147	PC	1	SF	366	0.5171	F	2	2	2	1	0		2	2	3	3	2			3	2		2			0	0	0	147
118	93H36-	149	PC	1	SF	366	0.2017	F	1	1	2	1	0		1	2	0					3	3					1	0	0	149
119	93H36-	150	PC	1	SF	366	0.5525	M	2	1	2	1	1			2	0		2			3	2					1	1	1	150
120	93H36-	152	PC	1	SF	366	0.5059	F	1	1	2	1	0		1	3	0			1		2	2		2			1	0	0	152
121	93H36-	1	FA	3	SF	366	0.4153	F	1	2	1	1	0		2	2	0					2	1					0	1	0	1
122	93H36-	5	FA	3	SF	366	0.388	F	2	0	2	1	0		2	2	1					2	1					1	0	1	5

APPENDIX I
AFB1-92: 6-MONTH SAMPLES

#	C#	S#	Exposure Diet	Exposure Conc.	FIX	AGE	Weight (g)	SE	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	FCA	NEO	MBE	S#
123	93H36-	16	FA	3	SF	366	0.4004	M	1	3	1	1	0			2	0		3			0	2					1	1	0	16
124	93H36-	22	FA	3	SF	366	0.212	F	1	0	1	1	0		1	2	0		1			0	2					0	1	0	22
125	93H36-	26	FA	3	SF	366	0.355	M	2	2	2	1	0			NP	0					2	2					1	0	1	26
126	93H36-	37	FA	3	SF	366	0.388	M	2	2	2	1	2			2	3					2	2					1	0	0	37
127	93H36-	51	FA	3	SF	366	0.405	M	2	0	2	1	0			2	0					2	2					1	0	1	51
128	93H36-	52	FA	3	SF	366	0.259	F	3	3	1	1	0		1	1	0					3	2					1	0	1	52
129	93H36-	54	FA	3	SF	366	0.171	F	1	0	1	1	0		1	1	0		3			2	2					1	0	1	54
130	93H36-	67	FA	3	SF	366	0.3456	M	2	2	3	1	0			2	2					2	2					1	0	1	67
131	93H36-	68	FA	3	SF	366	0.4963	F	1	0	2	1	0		3	2	0		2			2	2					0	1	0	68
132	93H36-	93	FA	3	SF	366	0.3784	F	1	2	1	1	1		2	3	3		3	3		2	3				2	0	1	0	93
133	93H36-	105	FA	3	SF	366	0.6187	M	1	2	3	1	0			2	3	2		2		2	1		1	1		1	0	1	105
134	93H36-	111	FA	3	SF	366	0.274	F	1	1	1	1	0		2	2	3			2		2	2					0	1	0	111
135	93H36-	115	FA	3	SF	366	0.3525	M	2	1	1	1	0			2	3					2	2					0	1	0	115
136	93H36-	118	FA	3	SF	366	0.3306	M	1	1	2	1	0			2	3					3	2					0	0	1	118
137	93H36-	126	FA	3	SF	366	0.5006	F	1	3	1	1	0		1	3	3					3	3					1	0	1	126
138	93H36-	133	FA	3	SF	366	0.267	F	1	1	2	1	0		1	1	3			2		2	1					0	1	0	133
139	93H36-	142	FA	3	SF	366	0.1835	F	1	2	2	1	0	3	2	2	2		2	3		3	3	2	3			1	0	0	142
140	93H36-	146	FA	3	SF	366	0.4076	F	1	2	3	1	3		2	3	3			2		3	2				3	1	1	0	146
141	93H36-	76	PC	3	SF	366	0.3708	M	1	2	1	1	3			3	0		1			2	2					0	0	0	76
142	93H36-	2	PC	3	SF	366	0.544	M	3	2	2	1	0			1	0					2	2				2	1	0	1	2
143	93H36-	6	PC	3	SF	366	0.645	F	1	0	2	1	0		2	3	4		3	3		1	2					0	1	0	6
144	93H36-	18	PC	3	SF	366	0.353	M	1	0	2	1	0			3	0					3	2					1	0	1	18
145	93H36-	21	PC	3	SF	366	0.379	F	1	1	3	1	0		2	0	0					4	2					1	0	1	21
146	93H36-	24	PC	3	SF	366	0.492	M	1	2	2	1	2			3	0					2	0					1	0	1	24
147	93H36-	27	PC	3	SF	366	0.283	U	2	2	2	1	1			2	0	2				3	0					0	0	1	27
148	93H36-	28	PC	3	SF	366	0.3663	M	1	1	3	1	0			2	1		1			2	2				1	0	0	0	28
149	93H36-	35	PC	3	SF	366	0.3911	F	1	1	2	1	2		2	2	0	3				1	1					0	1	0	35
150	93H36-	39	PC	3	SF	366	0.5681	M	1	1	1	1	0			2	3					1	2				2	1	1	0	39
151	93H36-	48	PC	3	SF	366	0.2831	M	2	2	2	1	0			1	0					2	2					0	0	1	48
152	93H36-	50	PC	3	SF	366	0.4721	F	1	1	2	1	2		2	2	0	2	1			2	2					1	0	0	50
153	93H36-	61	PC	3	SF	366	0.5098	F	1	3	1	1	2		2	2	2	2	2	3		3	2				2	1	1	0	61
154	93H36-	72	PC	3	SF	366	0.5843	M	2	0	3	1	1			2	0					1	1					1	0	1	72
155	93H36-	83	PC	3	SF	366	0.5221	M	1	1	2	1	4			1	0		3			0	0					0	0	0	83
156	93H36-	103	PC	3	SF	366	0.433	F	1	2	1	1	0		2	2	1	2		3		1	1		3			1	1	0	103
157	93H36-	140	PC	3	SF	366	0.659	M	1	2	2	1	4			2	0		2	2		2	1					1	0	0	140
158	93H36-	151	PC	3	SF	366	0.4823	M	1	1	3	1	2			2	0			1		2	2					0	0	1	151
159	93H36-	153	PC	3	SF	366	0.4831	M	1	1	2	1	0			2	0					1	1					1	0	1	153
160	93H36-	154	PC	3	SF	366	0.4789	M	1	0	2	1	0			2	0					1	1					1	0	1	154

Exposure													Frequency		
Diet	Conc.	N		Weight (g)	HG	VE	Atly	DM	APH	SH	HMA	RMA	FCA	NEO	MBE
FA	0	20	mean	0.354	1.0	1.1	2.4	0.7	1.5	0.6	0.8	1.9	0.15	0	0
			SE	0.019	0.00	0.23	0.11	0.24	0.20	0.21	0.16	0.11			
PC	0	20	mean	0.445	1.4	1.4	2.2	0.8	2.1	0.1	0.9	1.6	0.1	0	0
			SE	0.042	0.13	0.17	0.17	0.25	0.17	0.10	0.20	0.17			
FA	0.3	20	mean	0.379	1.9	1.1	2.3	0.2	1.4	0.8	1.8	2.0	0.55	0.05	0.15
			SE	0.022	0.16	0.20	0.15	0.12	0.17	0.22	0.19	0.11			
PC	0.3	20	mean	0.495	1.1	1.7	2.3	0.7	2.2	0.2	1.5	1.7	0.2	0.05	0.05
			SE	0.023	0.07	0.27	0.14	0.26	0.12	0.08	0.21	0.13			
FA	1	20	mean	0.404	1.7	1.3	2.2	0.5	1.6	0.7	1.6	1.9	0.55	0.05	0.6
			SE	0.036	0.15	0.19	0.15	0.21	0.17	0.23	0.15	0.10			
PC	1	20	mean	0.432	1.5	1.4	2.2	0.5	2.2	0.7	2.1	1.9	0.65	0.15	0.25
			SE	0.026	0.15	0.22	0.11	0.18	0.14	0.25	0.14	0.11			
FA	3	20	mean	0.357	1.4	1.5	1.7	0.3	2.0	1.6	2.1	2.0	0.6	0.45	0.45
			SE	0.025	0.13	0.23	0.16	0.18	0.13	0.32	0.18	0.14			
PC	3	20	mean	0.465	1.3	1.3	2.0	1.2	2.0	0.6	1.8	1.4	0.6	0.25	0.5
			SE	0.024	0.12	0.19	0.15	0.31	0.17	0.26	0.21	0.17			

			AFB1 Concentration (ppm)			
		Diet	0	0.3	1	3
Weight (g)	mean	FA	0.353	0.379	0.403	0.357
			0.019	0.022	0.035	0.024
	mean	PC	0.445	0.495	0.432	0.465
			0.042	0.023	0.026	0.024
HG	mean	FA	1	1.9	1.7	1.4
			0.000	0.161	0.147	0.134
	mean	PC	1.4	1.1	1.45	1.25
			0.134	0.069	0.153	0.123
VE	mean	FA	1.1	1.05	1.25	1.45
			0.228	0.198	0.190	0.235
	mean	PC	1.4	1.65	1.4	1.25
			0.169	0.274	0.222	0.190
	N	FA	20	20	20	20
	freq.		0.3	0.15	0.25	0.15
DM	mean		0.65	0.2	0.5	0.3
			0.244	0.117	0.212	0.179
	N	PC	20	19	20	20
	freq.		0.4	0.315	0.3	0.5
	mean		0.8	0.736	0.5	1.15
			0.247	0.256	0.185	0.310
APH	mean	FA	1.45	1.4	1.55	2
			0.198	0.169	0.170	0.129
	mean	PC	2.053	2.211	2.2	1.95
			0.174	0.120	0.138	0.170
SH	mean	FA	0.55	0.8	0.7	1.6
			0.211	0.225	0.231	0.320
	mean	PC	0.1	0.15	0.7	0.55
			0.100	0.082	0.252	0.256
HMA	mean	FA	0.75	1.8	1.55	2.05
			0.160	0.186	0.153	0.185
	mean	PC	0.9	1.5	2.1	1.8
			0.204	0.212	0.143	0.213
FCA	(# examined)		20	20	20	20
	freq.	FA	0.15	0.55	0.55	0.6
	(# w/ foci)		3	11	11	12
	(# examined)		20	20	20	20
	freq.	PC	0.1	0.2	0.65	0.6
	(# w/ foci)		2	4	13	12
NEO	(# examined)		20	20	20	20
	freq.	FA	0	0.05	0.05	0.45
	(# w/ neoplasia)		0	1	1	9
	(# examined)		20	20	20	20
	freq.	PC	0	0.05	0.15	0.25
	(# w/ neoplasia)		0	1	3	5
			0.000			
MBE	freq.	FA	0	0.15	0.6	0.45
	freq.	PC	0	0.05	0.25	0.5

APPENDIX II

Processing: fish were cassetted, fixed in Bouin's (6-7-93 through 6-9-93), transected midsagittaly and transferred to 70% EtOH about 24 h after fixation)

HSI = hepatosomatic index [(liver wt./body wt.)*100]

8- Sex, M= male, F= female, NP = gonad not present

9- (HG) Hepatic glycogen/vacuolation 1=min. 2=mod. 3= abund. NP= liver not present, A= autolyzed

10- (VE) Vacuolar encephalopathy 0=none 1=min 2=mild 3=mod 4=severe, NP , A

11- (A) Autolysis; 1=min, 2=mild, 3=mod, 4=sev

12- Lesions (LES); 0=no histologic lesions; 1=lesions present

STANDARD LESION SCORES: 1 = minimum (min), 2 = mild, 3 = moderate (mod), 4 = severe (sev)

13- (DM) Disseminated Mycobacteriosis;

14- (GPC) Granulomatous pericholangitis; old abbreviation = MS (malignant spongiosis)

15- (OG) Granulomatous oophoritis;

16- (APH) Atrial phagocyte hypertrophy;

17- (SH or HS) Spongiosis hepatis;

18- (GGH) Gas gland hyperplasia;

19- (MDN) Myofiber degeneration and/or necrosis;

20- (HN) Hepatic necrosis; individual cell or zonal;

21- (ENT) enteritis;

22- (HMA) Hepatic macrophage aggregates;

23- (RMA) renal macrophage aggregates;

24- (FLK) Flukes or monogenetic trematodes;

25- (GLH) gill lamellar epithelial hyperplasia

26- GLT (gill lamellar telangiectasis)

27- HFC (hepatic fatty change)

28 - (MBE) mixed basophilic and eosinophilic hepatocytes

29- (FCA) foci of cellular alteration, liver; P = present(1); A = absent (0);(TYP) type =
aph = amphophilic
bph = basophilic
eph = eosinophilic (no granules or droplets)
epr = eosinophilic, proteinaceous
egr = eosinophilic, granular (droplets)
fom = foamy (small fat droplets)
gly = glycogen-rich (clear cell)
vac = vacuolated (large lipid droplets)

30- (NEO) Neoplasia; P = present; see "COMMENTS" for type.
(TYP) Types (HAD) hepatocellular adenoma

31- (LBO) liver tissue is the body sections, so liver wt. probably underestimated

32- (XXX) other lesions; see under "comment"
FCH (fibrocartilaginous hyperplasia, mandible)
RTN (renal tubular necrosis)

#	Rep.	Exposure Diet	Conc.	Date Sampled	Block #	Body Wt. (g)	Liver Wt. (g)	HSI	LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	TYPE aph	bph	eph	epr	egr	fom	gly	vac	NEO	TYP
1	1	FA	0	06/07/93	93H47- 164	.541	.017	3.14		NP	3	2	1	0	0	0		NP	0				0	2					0	0										0
2	2	FA	0	06/07/93	93H47- 184	.378	.011	2.91		F	1	0	1	1	3	0	1	2	2		1		2	2					0	0										0
3	3	FA	0	06/07/93	93H47- 43	.577	.034	5.89		F	2	2	1	1	2	0	1	2	3		2		1	2					0	0										0
4	4	FA	0	06/07/93	93H47- 145	.438	.010	2.28		M	1	1	1	1	1	0		NP	0				3	2					0	1	0	0	0	0	0	2	0	0		0
5	5	FA	0	06/07/93	93H47- 160	.609	.011	1.81		M	2	2	1	1	0	0		NP	2				2	2					0	1	0	0	0	0	0	3	0	0		0
6	6	FA	0	06/07/93	93H47- 88	.422	.010	2.37	1	M	1	1	1	1	2	0		NP	1	3			1	2					0	0										0
7	7	FA	0	06/07/93	93H47- 107	.648	.016	2.47		F	3	2	1	1	0	0	2	NP	2				1	2					0	0										0
8	8	FA	0	06/07/93	93H47- 27	.557	.029	5.21		M	3	0	1	1	2	0		2	0	1			0	2					0	0										0
9	9	FA	0	06/07/93	93H47- 31	.566	.012	2.12		M	1	1	2	1	3	0		NP	0	2			1	1					0	0										0
10	10	FA	0	06/07/93	93H47- 22	.416	.012	2.88		NP	1	3	1	1	2	0		NP	0				1	1					0	1	0	0		0	2	1	0	0		0
11	11	FA	0	06/07/93	93H47- 185	.378	.007	1.85		F	2	1	1	1	1	0	NP	NP	0				1	1					0	0										0
12	12	FA	0	06/07/93	93H47- 75	.554	.012	2.17		M	1	NP	1	1	0	0		NP	0	1			1	2					0	1	0	0		0	1	0	1	0		0
13	13	FA	0	06/07/93	93H47- 94	.678	.010	1.47		M	1	1	1	1	1	0		1	0				3	2					0	0										0
14	14	FA	0	06/07/93	93H47- 87	.418	.006	1.44	1	NP	1	1	1	1	3	0		2	0	3			0	1					0	0										0
15	15	FA	0	06/07/93	93H47- 137	.360	.012	3.33		M	2	1	1	1	2	0		2	0	2			1	2					0	0										0

#	Rep.	Exposure Diet	Conc.	Date Sampled	Block #	Body Wt. (g)	Liver Wt. (g)	HSI	LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	TYPE aph	bph	eph	epr	egr	fom	gly	vac	NEO	TYP
16	16	FA	0	06/07/93	93H47- 98	.338	.010	2.96		F	3	1	2	1	0	0	NP	0	0				2	2					0	1	0	0	0	0	4	0	0	0	0		
17	17	FA	0	06/07/93	93H47- 5	.601	.017	2.83		F	2	4	1	1	2	0	2	NP	2			2	2	2					0	1	0	0		0	0	3	0	0	0	
18	18	FA	0	06/07/93	93H47- 127	.533	.013	2.44		M	2	2	1	1	0	2		NP	1				3	2					0	0									0	
19	19	FA	0	06/07/93	93H47- 197	.600	.021	3.50		M	3	2	2	1	2	0		2	1				1	2					0	1	0	0	0	0	1	0	0	0	0	
20	20	FA	0	06/07/93	93H47- 114	.299	.005	1.67		F	1	2	1	1	0	0	2	NP	3				2	NP					0	0									0	
21	21	FA	0	06/09/93	93H47- 128	.505	ND			M	1	2	2	1	2	0		2	2			2	2	2					0	1	0	0	0	0	1	0	1	0	0	
22	22	FA	0	06/09/93	93H47- 163	.553	ND			F	3	0	3	1	0	0	2	1	0				0	2					O	0									0	
23	23	FA	0	06/09/93	93H47- 55	.886	ND			F	3	1	3	1	2	0	2	2	0	2				2	2					0	1	0	0		0	0	0	1	0	0	
24	24	FA	0	06/09/93	93H47- 13	.684	ND			F	3	2	2	1	2	0	2	2	0			2	1	2					0	1	1	0		0	0	0	1	0	0	
25	25	FA	0	06/09/93	93H47- 148	.214	ND			F	1	2	2	1	0	0	1	2	0				1	2					0	0									0	
26	26	FA	0	06/09/93	93H47- 20	.662	ND			M	1	0	2	1	3	0		1	2			2	0	1				3	0	1	0	0		0	1	0	0	0	0	
27	27	FA	0	06/09/93	93H47- 25	.464	ND			F	1	0	3	1	2	0	1	1	0				1	2					0	0									0	
28	28	FA	0	06/09/93	93H47- 37	.608	ND			M	1	2	3	1	2	0		1	0				0	2				2	0	0									0	
29	29	FA	0	06/09/93	93H47- 100	ND	ND			F	2	1	2	1	0	2	2	2	1				1	2					0	0									0	
30	30	FA	0	06/09/93	93H47- 125	.457	ND			M	2	2	2	1	2	0		1	0			2	1	2					0	0									0	
31	31	FA	0	06/09/93	93H47- 131	.526	ND			M	3	2	2	1	2	0		2	2			2	1	1					0	1	0	0	0	0	0	0	0	1	0	
32	32	FA	0	06/09/93	93H47- 201	.586	ND			F	3	2	3	1	1	0	1	2	2				1	2					0	0									0	
33	33	FA	0	06/09/93	93H47- 190	.603	ND			F	3	2	2	1	0	0	2	1	0				2	2					0	0									0	
34	34	FA	0	06/09/93	93H47- 80	.674	ND			M	2	0	2	1	2	0		2	0			2	1	2					0	1	0	0	0	0	0	0	0	10	0	
35	35	FA	0	06/09/93	93H47- 119	.419	ND			M	3	1	3	1	2	0		2	0			3	1	2					0	0									0	
36	36	FA	0	06/09/93	93H47- 33	.774	ND			F	2	3	3	1	2	0	2	3	1				2	2					0	0									0	
37	37	FA	0	06/09/93	93H47- 81	.505	ND			F	1	1	2	1	2	0	3	2	3				1	2					0	0									0	
38	38	FA	0	06/09/93	93H47- 147	.464	ND			F	1	2	3	1	0	0	1	1	0				1	2					0	0									0	
39	39	FA	0	06/09/93	93H47- 189	.295	ND			F	2	0	2	1	2	0	2	2	2				2	2					0	0									0	
40	40	FA	0	06/09/93	93H47- 155	.679	ND			F	3	3	2	1	2	0	3	2	0			2	1	2		2			0	0									0	
41	41	FA	0	06/09/93	93H47- 92	.659	ND			F	3	3	3	1	2	0	2	1	0				2	2					0	0									0	
42	42	FA	0	06/09/93	93H47- 168	.502	ND			M	3	2	3	1	1	0		2	0				1	2					0	0									0	
43	43	FA	0	06/09/93	93H47- 180	.837	ND			F	3	1	3	1	2	0	2	3	2	2	2		1	2					0	0	0	0	0	0	0	0	0	1	0	
44	44	FA	0	06/09/93	93H47- 187	.556	ND			F	2	1	2	1	2	0	2	1	0				2	3					0	0									0	
45	45	FA	0	06/09/93	93H47- 144	.576	ND			M	3	2	3	1	0	0		0	0				0	2					0	0									0	
46	46	FA	0	06/09/93	93H47- 91	.399	ND			M	2	0	3	1	2	0		0	2				2	2					0	0									0	
47	47	FA	0	06/09/93	93H47- 156	.427	ND			M	2	1	2	1	1	0		2	2			1	1	2					0	0									0	
48	48	FA	0	06/09/93	93H47- 105	.300	ND			M	3	3	3	1	0	0		1	2				1	2					0	0									0	
49	49	FA	0	06/09/93	93H47- 51	.708	ND			F	3	1	2	1	0	0	2	0	0	2	1		2	2					0	1	0	0		0	0	0	2	0	0	
50	50	FA	0	06/09/93	93H47- 56	.472	ND			M	1	2	2	1	3	0		2	2				2	3				3	0	0	0	0		0	0	0	0	1	0	
51	51	FA	0	06/09/93	93H47- 112	.567	ND			F	3	1	2	1	0	0	2	1	0				2	2					0	1	0	0	0	0	0	0	0	1	0	
52	52	FA	0	06/09/93	93H47- 89	.308	ND			M	1	2	2	1	2	0		2	4				3	2				2	0	0									0	
53	53	FA	0	06/09/93	93H47- 7	.497	ND			M	3	4	2	1	2	0		0	3			2	0	2					1	0									0	
54	54	FA	0	06/09/93	93H47- 120	.381	ND			F	3	0	2	1	1	0	2	1	0			1	2	2					0	0									0	
55	55	FA	0	06/09/93	93H47- 118	.631	ND		no slides																														1	GGC
56	56	FA	0	06/09/93	93H47- 133	.313	ND			F	1	3	2	1	2	0	2	2	0				1	3					0	0									0	
57	57	FA	0	06/09/93	93H47- 198	.667	ND			M	3	0	3	1	2	0		2	0			2	2	2					0	1	0	0	0	0	0	0	1	2	0	
58	58	FA	0	06/09/93	93H47- 167	.737	ND			M	3	2	3	1	0	0		1	0				1	2					0	0									0	
59	59	FA	0	06/09/93	93H47- 111	.442	ND			M	2	3	2	1	0	0		2	0				1	1				2	0	0									0	
60	60	FA	0	06/09/93	93H47- 122	.439	ND			M	2	1	2	1	2	0			3			2	2	3															
61	61	FA	0	06/09/93	93H47- 130	.476	ND			F	3	0	3	1	2	0	2	2	2				3	2					0	1	0	0	0	0	0	3	0	0	0	
62	62	FA	0	06/09/93	93H47- 78	.347	ND			F	1	1	2	1	1	3	2	2	2			2	2	2					0	0		HERE							0	
63	63	FA	0	06/09/93	93H47- 28	.460	ND			F	2	1	2	1	0	0	2	0	0				1	2					0	0									0	
64	64	FA	0	06/09/93	93H47- 142	.742	ND			F	3	1	3	1	0	0	1	2	0				2	3					0	0									0	

#	Rep.	Exposure Diet	Conc.	Date Sampled	Block #	Body Wt. (g)	Liver Wt. (g)	HSI	LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	TYPE aph	bph	eph	epr	egr	fom	gly	vac	NEO	TYP
65	65	FA	0	06/09/93	93H47- 90	.627	ND			F	3	1	3	1	0	0	2	1	1				1	2					0	0									0	
66	66	FA	0	06/09/93	93H47- 96	.380	ND			F	2	2	2	1	0	0	2	2	0				1	2					0	0									0	
67	67	FA	0	06/09/93	93H47- 177	.493	ND			M	2	2	1	1	2	0		2	2		1		0	1					0	1	0	0	0	0	1	6	0	0	0	
68	68	FA	0	06/09/93	93H47- 110	.567	ND			M	3	1	3	1	2	0		2	2		2		1	2					0	0									0	
69	69	FA	0	06/09/93	93H47- 59	.464	ND			F	2	1	2	1	2	0	2	2	0		2		1	2					0	0									0	
70	70	FA	0	06/09/93	93H47- 4	.550	ND			M	2	3	2	1	2	0		1	3				2	3					0	0									0	
71	71	FA	0	06/09/93	93H47- 58	.182	ND			F	1	1	2	1	0	0	1	1	2				1	2					0	0									0	
72	72	FA	0	06/09/93	93H47- 57	.300	ND			M	1	1	2	1	0	0		2	0				2	3					0	0									0	
73	73	FA	0	06/09/93	93H47- 115	.478	ND			F	1	2	2	1	2	0	2	2	2		1		3	2					0	0									0	
74	74	FA	0	06/09/93	93H47- 48	.168	ND			F	3	1	2	1	0	0	1	1	0				1	2					0	0									0	
75	75	FA	0	06/09/93	93H47- 116	.500	ND			F	3	3	2	1	2	0	2	2	0		2		1	2					0	0									0	
76	76	FA	0		93H47- 140	not used				M	2	1	2	0	0	0		2	0				1	2					0	0									0	
77	77	FA	0		93H47- 104	not used																																		
78	78	FA	0		93H47- 117	not used																																		
79	79	FA	0		93H47- 170	not used																																		
80	80	FA	0		93H47- 157	not used																																		
81	81	FA	0		93H47- 86	not used																																		
82	82	FA	0		93H47- 8	not used																																		
83	83	FA	0		93H47- 159	not used																																		
84	84	FA	0		93H47- 85	not used																																		
85	85	FA	0		93H47- 193	not used																																		
86	1	PC	0	06/07/93	93H47- 15	.938	.013	1.39		F	3	0	2	1	0	0	3	2	0				1	2				1	0	1	0	0		0	0	0	0	6	0	
87	2	PC	0	06/07/93	93H47- 14	.732	.023	3.14		F	3	3	2	1	0	0	2	NP	0				1	0					0	0									0	
88	3	PC	0	06/07/93	93H47- 47	.777	.027	3.47	1	F	1	1	1	1	4	0	2	3	0				2	2					0	0									0	
89	4	PC	0	06/07/93	93H47- 95	.592	.030	5.07	1	F	3	4	1	1	2	0	2	NP	0	2			3	2					0	0									0	
90	5	PC	0	06/07/93	93H47- 186	.900	.039	4.33		M	3	1	2	1	2	0		NP	0	2			1	2					0	0									0	
91	6	PC	0	06/07/93	93H47- 158	.863	.019	2.20	1	M	3	2	2	1	3	0		1	0	2			0	2					O	0									0	
92	7	PC	0	06/07/93	93H47- 176	.273	ND			F	1	1	1	1	0	0	2	2	NP				NP	2					0	0									0	
93	8	PC	0	06/07/93	93H47- 60	.768	.027	3.52		F	3	1	2	1	2	0	1	2	0				2	1					0	1	0	0		0	0	0	0	1	0	
94	9	PC	0	06/07/93	93H47- 188	.380	.014	3.68		F	2	1	2	1	0	0	2	3	0				2	2					0	0									0	
95	1	FA	.3	06/07/93	93H47- 97	.743	.021	2.83	1	M	3	1	2	1	0	0		2	0				2	2	2	2			0	1	1	0	0	0	1	0	0	2	0	
96	2	FA	.3	06/07/93	93H47- 79	.652	.017	2.61	1	NP	3	1	1	1	0	0		NP	0				2	1					0	1	0	1	1	0	1	1	0	0	0	
97	3	FA	.3	06/07/93	93H47- 46	.680	.021	3.09	1	M	2	1	1	1	2	3		1	2				2	2					0	0									0	
98	4	FA	.3	06/07/93	93H47- 2	.307	.003	.98		F	1	1	3	1	0	0	2	NP	0				3	NP				3	0	0									0	
99	5	FA	.3	06/07/93	93H47- 42	.962	.110	11.43		F	2	2	1	1	3	0	2	0	3				2	2					0	1	1	2		0	0	0	0	0	0	
100	6	FA	.3	06/07/93	93H47- 121	.391	.023	5.88		M	NP	4	1	1	0	NP		NP	NP		1		NP	2					NP	NP									NP	
101	7	FA	.3	06/07/93	93H47- 49	.510	.015	2.94		NP	2	1	1	0	0	0		NP	0				1	3					0	1	0	0		0	0	6	0	0	0	
102	8	FA	.3	06/07/93	93H47- 134	.528	.017	3.22		M	3	3	1	1	0	0		NP	0				1	2					0	1	1	0	0	0	0	0	0	0	0	
103	9	FA	.3	06/07/93	93H47- 199	.461	.011	2.39		NP	3	3	1	1	0	0		1	0				2	1					0	1	0	0	0	3	1	0	0	0	0	
104	10	FA	.3	06/07/93	93H47- 32	.668	.018	2.69		M	3	1	1	1	0	0		2	1				2	2	2	2			0	0									0	
105	11	FA	.3	06/07/93	93H47- 52	.572	.042	7.34		F	2	1	2	1	0	0	2	1	2				1	2				3	0	1	4	1		1	1	0	0	0	0	
106	12	FA	.3	06/07/93	93H47- 73	.736	.080	10.87		F	1	2	1	1	0	0	3	0	4				1	2				4	0	1	0	11		0	0	0	0	1	0	
107	13	FA	.3	06/07/93	93H47- 67	.674	.055	8.16	1	F	2	1	2	1	2	0	2	1	0	2			2	2				2	0	1	0	0		2	0	0	0	2	0	
108	14	FA	.3	06/07/93	93H47- 34	.342	.012	3.51		M	2	1	1	1	0	2		NP	2				2	3					0	0									0	
109	15	FA	.3	06/07/93	93H47- 44	.592	.018	3.04	1	F	3	0	1	1	0	0	2	NP	0		2		3	2					0	1	1	1		0	2	0	0	0	1	HAD
110	16	FA	.3	06/07/93	93H47- 36	.382	.012	3.14		F	3	1	2	1	0	0	2	NP	0	2			2	2					0	1	0	0		1	1	0	0	0	0	
111	17	FA	.3	06/07/93	93H47- 50	.820	.023	2.80	1	M	0	0	1	1	0	0		3	0				1	1				4	0	1	8	0		7	0	0	0	0	1	HAD
112	18	FA	.3	06/07/93	93H47- 9	.479	.011	2.30		M	2	2	1	1	0	0		NP	0				1	2					1	1	0	0		0	3	5	0	2	0	
113	19	FA	.3	06/07/93	93H47- 135	.294	.013	4.42	1	F	1	2	1	1	0	0	2	2	2		2		3	3					0	1	0	3	0	0	0	0	0	0	1	HAD(2

#	Rep.	Exposure Diet	Exposure Conc.	Date Sampled	Block #	Body Wt. (g)	Liver Wt. (g)	HSI	LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	TYPE aph	bph	eph	epr	egr	fom	gly	vac	NEO	TYP
114	20	FA	.3	06/07/93	93H47- 123	.300	.007	2.33		M	1	2	1	1	0	0		1	0					3	2					0	0									0	
115	21	FA	.3	06/07/93	93H47- 17	.404	.020	4.95		NP	2	1	1	1	0	0		NP	0					2	2					0	0									0	
116	22	FA	.3	06/07/93	93H47- 10	.668	.027	4.04		M	2	1	1	1	0	0		NP	0					2	2					0	1	1	0		0	0	4	0	0	0	
117	23	FA	.3	06/07/93	93H47- 12	.370	.020	5.41		M	3	2	1	1	0	0		NP	3					3	2					0	0									0	
118	24	FA	.3	06/07/93	93H47- 161	.548	.014	2.55	1	M	2	1	1	1	0	0		1	0					2	2					0	1	0	0	0	1	0	1	0	1	0	
119	25	FA	.3	06/07/93	93H47- 74	.232	.013	5.60		M	1	1	1	1	0	0		NP	2					3	2					0	1	1	0		0	1	0	0	0	0	
120	26	FA	.3	06/07/93	93H47- 169	.455	.023	5.05		NP	2	0	1	1	0	0		1	2					2	1					0	1	0	0	0	0	0	0	2	0	1	HCA
121	27	FA	.3	06/07/93	93H47- 192	.425	.020	4.71		F	1	1	1	1	3	0	2	1	3					2	2					0	0									0	
122	28	FA	.3	06/07/93	93H47- 108	not used																																			
123	29	FA	.3	06/07/93	93H47- 101	not used																																			
124	30	FA	.3	06/07/93	93H47- 68	not used																																			
125	1	PC	.3	06/07/93	93H47- 66	.855	.029	3.39		F	3	1	1	1	2	0	2	NP	1	2				2	2					0	0									0	
126	2	PC	.3	06/07/93	93H47- 151	.576	.022	3.82		F	3	0	1	1	0	2	2	2	2					2	2					0	0									0	
127	3	PC	.3	06/07/93	93H47- 76	.755	.025	3.31		M	3	2	1	1	2	0		1	0					2	1					0	0									0	
128	4	PC	.3	06/07/93	93H47- 175	.880	.018	2.05		NP	2	0	2	1	1	0		NP	0					2	2					0	0									0	
129	5	PC	.3	06/07/93	93H47- 149	1.136	.045	3.96		F	3	4	2	1	0	0	2	2	0	3	2			1	2					0	0									0	
130	6	PC	.3	06/07/93	93H47- 63	.636	.012	1.89		M	2	3	1	1	3	0		2	0					1	2					0	1	0	0		0	0	0	0	1	0	
131	7	PC	.3	06/07/93	93H47- 129	.394	.016	4.06		M	2	2	1	1	0	0		NP	0					2	2					0	1	0	0	0	0	0	0	0	3	0	
132	8	PC	.3	06/07/93	93H47- 165	.718	.029	4.04	1	F	3	4	2	1	1	0	1	2	0					2	2					0	1	0	0	0	0	0	0	1	0	0	
133	9	PC	.3	06/07/93	93H47- 21	.445	.012	2.70		M	3	1	1	1	0	0		1	0					1	1					0	0									0	
134	10	PC	.3	06/07/93	93H47- 103	.824	.032	3.88		F	3	0	2	1	0	0	0	2	0					2	2					0	0									0	
135	11	PC	.3	06/07/93	93H47- 38	.742	.026	3.50		F	3	3	2	1	2	0	2	NP	2					3	2					0	1	0	0		0	0	0	1	0	0	
136	12	PC	.3	06/07/93	93H47- 153	.565	.033	5.75		F	1	2	1	1	3	0	3	2	2	3	2	2		3	1				2	0	1	0	0	0	0	0	1	0	1	1	HCA
137	13	PC	.3	06/07/93	93H47- 106	.580	.018	3.10	1	M	2	2	2	1	1	0		2	1	2				2	2					0	0									0	
138	14	PC	.3	06/07/93	93H47- 143	.977	.037	3.79		F	3	1	1	1	0	0	3	NP	2					2	2					0	0									0	
139	15	PC	.3	06/07/93	93H47- 191	.482	.011	2.28		M	2	1	2	1	3	0		NP	2					2	2					0	0									0	
140	16	PC	.3	06/07/93	93H47- 62	.517	.030	5.80	1	F	3	1	1	1	0	0	2	2	0					2	2					0	1	0	0		0	1	0	0	0	0	
141	1	FA	1	06/08/93	93H47- 182	.491	.029	5.91		F	1	1	1	1	2	0	2	NP	0		2	2		2	3					0	1	0	1	0	0	0	0	0	0	1	HCA
142	2	FA	1	06/08/93	93H47- 179	.355	.005	1.41		M	2	1	1	1	0	0		2	0	3	1			3	2					0	0									0	
143	3	FA	1	06/08/93	93H47- 61	.683	.064	9.37		M	1	4	2	1	0	0		0	0		3	4	2	1	2					0	1	0	0		1	0	0	0	0	1	HCA
144	4	FA	1	06/08/93	93H47- 136	.167	ND			NP	1	1	3	1	0	0		2	0		2			3	3					0	0									0	
145	5	FA	1	06/08/93	93H47- 45	.797	.020	2.51	1	M	2	0	2	1	0	0		NP	3					2	2					0	1	0	3		0	3	0	0	1	1	HCA
146	6	FA	1	06/08/93	93H47- 77	.738	.025	3.39		M	3	0	1	1	2	0		NP	0	3				3	2					0	1	0	0	BEGIN	0	2	3	0	1	0	
147	7	FA	1	06/08/93	93H47- 124	.250	.002	.60		M	2	1	1	1	0	0		2	0		2			2	2					0	0									0	
148	8	FA	1	06/08/93	93H47- 54	.936	.099	10.58	1	M	1	0	2	1	0	0		1	0					2	2				4	0	1	3	0		0	1	0	0	0	1	HCA
149	9	FA	1	06/08/93	93H47- 200	.811	.045	5.55		M	2	2	1	1	0	0		NP	0					1	2					0	1	0	0	0	0	1	3	0	0	0	
150	10	FA	1	06/08/93	93H47- 39	.604	.018	2.98		M	3	0	2	1	0	0		2	0					1	2					0	1	0	0		0	0	1	0	0	0	
151	11	FA	1	06/08/93	93H47- 196	.627	.020	3.19		M	2	2	1	1	0	0		NP	2					1	1					0	1	2	0	0	0	1	1	0	0	0	
152	12	FA	1	06/08/93	93H47- 19	.588	.018	3.06	1	M	2	2	1	1	0	0		NP	3					0	2				3	0	0								1	HCA
153	13	FA	1	06/08/93	93H47- 16	.544	.033	6.07		M	3	2	1	1	0	0		NP	0					0	1					0	1	0	0		1	1	0	0	0	0	
154	14	FA	1	06/08/93	93H47- 195	.688	.020	2.91		F	1	0	1	1	0	0	2	1	0					1	1					0	1	0	1	0	0	0	1	0	1	0	
155	15	FA	1	06/08/93	93H47- 174	.445	.019	4.27		F	1	2	2	1	0	0	2	2	0					3	2					0	0									0	
156	16	FA	1	06/08/93	93H47- 166	.550	.030	5.45	1	M	3	1	1	1	1	0		1	2					3	2					0	1	0	0	0	0	1	0	0	0	0	
157	17	FA	1	06/08/93	93H47- 18	.530	.025	4.72		M	1	1	1	1	0	0		2	0	2				2	2					0	0									0	
158	18	FA	1	06/08/93	93H47- 30	.576	.034	5.90		NP	2	NP	1	1	0	0		2	0					1	NP					0	1	0	0		0	2	5	1	0	0	
159	19	FA	1	06/08/93	93H47- 93	.675	.022	3.26		M	2	NP	1	1	0	0		NP	2					2	2					0	1	0	0	0	1	0	0	1	1	0	
160	20	FA	1	06/08/93	93H47- 139	.377	.009	2.39		NP	2	NP	1	0	0	0		0	0					1	NP					0	0									0	
161	21	FA	1	06/08/93	93H47- 82	.580	.031	5.34		M	2	NP	1	1	0	0		NP	0					2	NP					0	1	0	0	0	0	1	0	0	0	0	
162	22	FA	1	06/08/93	93H47- 1	.428	.010	2.34		F	2	NP	1	1	0	0	2	NP	0					3	2					0	1	0	0		0	1	1	0	1	0	

#	Rep.	Exposure Diet	Exposure Conc.	Date Sampled	Block #	Body Wt. (g)	Liver Wt. (g)	HSI	LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	TYPE aph	bph	eph	epr	egr	fom	gly	vac	NEO	TYP
163	23	FA	1	06/08/93	93H47- 65	.415	.013	3.13		F	3	NP	1	1	0	0	0	NP	2				2	NP					0	0									0	
164	24	FA	1	06/08/93	93H47- 29	.720	.024	3.33		NP	1	1	1	1	0	0		NP	0				2	2					4	0	1	1	0		0	0	0	0	0	0	
165	25	FA	1	06/08/93	93H47- 23	.410	.007	1.71		F	2	NP	1	1	0	0	1	NP	0				2	NP						0	1	0	0		0	0	7	0	0	?	
166	26	FA	1	06/08/93	93H47- 70	.220	.014	6.36		F	2	NP	1	1	0	0	0	NP	0			2	4	NP						0	1	0	0		0	0	0	0	0	1	HAD
167	27	FA	1	06/08/93	93H47- 202	.560	.024	4.29		NP	2	NP	1	1	0	0		NP	0				2	NP						0	1	0	0	1	0	0	1	0	0	1	HAD
168	28	FA	1	06/08/93	93H47- 146	.587	.014	2.39		NP	3	NP	2	0	0	0		NP	0				1	NP						0	0									0	
169	29	FA	1	06/08/93	93H47- 72	.370	.014	3.78		NP	1	NP	1	1	0	0		NP	0				3	NP						0	0									1	HCA
170	30	FA	1	06/08/93	93H47- 11	.523	.040	7.65		F	2	NP	1	1	0	0	1	NP	3				3	NP						0	0									0	
171	31	FA	1	06/08/93	93H47- 40	.138	.002	1.45		F	1	NP	1	1	0	0	0	NP	2		2		3	2						0	0									0	
172	32	FA	1	06/08/93	93H47- 141	.516	.015	2.91		NP	3	NP	1	1	0	0		NP	0				2	NP						0	1	0	1	0	0	1	0	0	1	0	
173	33	FA	1	06/08/93	93H47- 178	not used																																			
174	1	PC	1	06/08/93	93H47- 126	.723	.024	3.32		F	2	NP	1	1	1	0	2	NP	0				2	NP						0	1	0	0	0	0	0	1	0	0	0	
175	2	PC	1	06/08/93	93H47- 150	.918	.030	3.27		NP	2	NP	1	1	2	0		NP	0				2	NP					3	0	1	0	0		0	0	0	0	3	0	
176	3	PC	1	06/08/93	93H47- 71	.608	.094	15.46		NP	2	NP	2	1	0	0		NP	0				1	NP						0	1	0	0		0	0	0	0	0	0	
177	4	PC	1	06/08/93	93H47- 132	.660	.020	3.03		NP	2	NP	1	1	0	0		NP	0			2	2	NP						0	1	0	0	0	0	1	1	0	0	0	
178	5	PC	1	06/08/93	93H47- 53	.616	.028	4.55		NP	2	NP	1	1	0	0		NP	0				1	1						0	1	0	0	0	0	0	0	0	1	0	
179	6	PC	1	06/08/93	93H47- 138	.786	.019	2.42		NP	3	NP	1	1	0	0		NP	0				2	NP						0	1	0	0	0	0	0	0	0	1	0	
180	7	PC	1	06/08/93	93H47- 83	.505	.013	2.57		M	2	4	2	1	2	0		2	0				2	1						0	0									0	
181	8	PC	1	06/08/93	93H47- 35	.560	.026	4.64		M	2	NP	2	1	0	0		NP	0				1	1						0	1	0	0		0	0	1	0	0	1	0	
182	9	PC	1	06/08/93	93H47- 194	not used																																			
183	1	FA	3	06/08/93	93H47- 162	.464	.040	8.62		M	2	0	1	1	0	0		2	3				2	2						0	1	0	1	0	0	0	0	0	1	0	
184	2	FA	3	06/08/93	93H47- 24	.414	.028	6.76		M	2	3	1	1	0	0		NP	0				1	1						0	0									1	HCA
185	3	FA	3	06/08/93	93H47- 181	.334	.011	3.29		M	1	1	1	1	0	0		1	0				2	1						0	0									0	
186	4	FA	3	06/08/93	93H47- 152	.572	.013	2.27		M	3	2	1	1	0	0		1	2				2	1					2	0	0								0	
187	5	FA	3	06/08/93	93H47- 26	.567	.015	2.65		M	2	NP	1	1	0	0		1	0				2	1						0	1	0	0		0	1	1	0	0	0	
188	6	FA	3	06/08/93	93H47- 171	.328	.020	6.10		M	3	0	1	1	0	0		NP	0				1	2						0	0									0	
189	7	FA	3		93H47- 154	not used																																			
190	8	FA	3		93H47- 3	not used																																			
191	9	FA	3		93H47- 113	not used																																			
192	10	FA	3		93H47- 109	not used																																			
193	11	FA	3		93H47- 41	not used																																			
194	12	FA	3		93H47- 102	not used																																			
195	1	PC	3	06/08/93	93H47- 84	.665	.037	5.56		M	2	4	1	1	0	0		NP	2	2			3	2						0	1	0	0	1	0	0	1	0	2	0	
196	2	PC	3	06/08/93	93H47- 69	.758	.021	2.77	1	M	1	4	1	1	0	0		NP	3		3	1	1	1						0	1	0	0		0	2	2	0	1	1	HCA
197	3	PC	3	06/08/93	93H47- 99	.550	.013	2.36		M	2	1	1	1	0	0		NP	0				2	1						0	1	0	0	0	0	0	3	0	7	0	
198	4	PC	3	06/08/93	93H47- 183	.542	.038	7.01		F	2	2	1	1	0	0	NP	NP	0				2	1						0	1	0	0	2	0	0	1	0	1	0	
199	5	PC	3	06/08/93	93H47- 6	.557	.018	3.23		M	2	3	2	1	1	2		2	0				2	2						0	1	0	0		0	1	0	1	0	0	
200	6	PC	3	06/08/93	93H47- 173	.523	.032	6.12		M	2	0	1	1	0	0		1	0				1	NP						0	0									0	
201	7	PC	3	06/08/93	93H47- 64	.700	.072	10.29		M	2	4	1	1	2	0		NP	0				1	1						0	1	0	0		0	2	1	0	3	0	
202	8	PC	3	06/08/93	93H47- 172	.574	.038	6.62		F	3	0	1	1	0	0	2	NP	0				2	1					3	0	1	1	2	1	0	0	0	0	0	1	HCA

NOTES: the foam is a BAD idea for fixation of liver; the hepatic architecture is SQUISHED, making reading difficult!!!

SUMMARY STATISTICS: Aflatoxin Army Study - sampled 3 months after aflatoxin feeding was stopped

Exposure																											TYPE									
Diet	Conc.	N	Statistic	Body Wt. (g)	Liver Wt. (g)	HSI	% LBO	SEX	HG	VE	Atly	LES	DM	GPC	OG	APH	SH	GGH	MDN	HN	ENT	HMA	RMA	FLK	GLH	GLT	HFC	MBE	FCA	aph	bph	eph	epr	egr	fom	gly	vac	NEO
FA	0	74	N	74		20	2		74	73	74	74	74	74	36	61	74	3	30			74	73		1		6	72	73	20	20	12	20	20	20	20	20	73
			mean	**.508**		**2.74**	**10**		**2.1**	**1.5**	**2.0**	**.99**	**1.3**	**.09**	**1.8**	**1.5**	**.92**	**2**	**1.8**			**1.4**	**2.0**		**2**		**2.3**	**.01**	**.25**	**.05**	**0**	**0**	**0**	**.55**	**.9**	**.35**	**.8**	**.01**
			±SE	.017		.25			.10	.12	.08	.01	.12	.05	.09	.09	.13	0	.11			.09	.05				.21	.01		.05	0	0	0	.22	.37	.13	.50	
PC	0	9	N	9		8	3		9	9	9	9	9	9	7	6	8	3				8	9				1	8	9	2	2		2	2	2	2	2	9
			mean	**.691**		**3.35**	**38**		**2.4**	**1.6**	**1.7**	**1**	**1.4**	**0**	**2**	**2.2**		**2**				**1.5**	**1.7**				**1**	**0**	**.22**	**0**	**0**		**0**	**0**	**0**	**0**	**3.5**	**0**
			±SE	.077		.41			.29	.41	.17	0	.50	0	.22	.31		0				.33	.24					0		0	0		0	0	0	0	2.5	
FA	.3	27	N	27		27	8		26	27	27	27	27	26	9	14	26	2	3			26	26		2	2	5	26	26	18	18	7	18	18	18	18	18	26
			mean	**.526**		**4.38**	**30**		**2**	**1.4**	**1.2**	**.96**	**.37**	**.19**	**2.1**	**1.2**	**1**	**2**	**1.7**			**2**	**2.0**		**2**	**2**	**3.2**	**.04**	**.69**	**1**	**1.1**	**.143**	**.83**	**.61**	**.94**	**.11**	**.44**	**.15**
			±SE	.035		.49			.17	.18	.10	.04	.18	.14	.11	.21	.25	0	.33			.14	.10		0	0	.37	.04		.47	.62	.143	.41	.20	.45	.11	.18	
PC	.3	16	N	16		16	3		16	16	16	16	16	16	9	10	16	4	2	1		16	16				1	16	16	6	6	3	6	6	6	6	6	16
			mean	**.693**		**3.58**	**19**		**2.6**	**1.7**	**1.4**	**1**	**1.1**	**.13**	**1.9**	**1.8**	**.75**	**2.5**	**2**	**2**		**1.9**	**1.8**				**2**	**0**	**.38**	**0**	**0**	**0**	**0**	**.17**	**.17**	**.33**	**.83**	**.06**
			±SE	.051		.28			.16	.33	.13	0	.30	.13	.31	.13	.23	.29	0			.14	.10					0		0	0	0	0	.17	.17	.21	.48	
FA	1	32	N	32		32	4		32	18	32	32	32	32	9	12	32	3	6	3	1	32	21				3	32	32	20	19	9	19	20	19	19	19	31
			mean	**.528**		**4.01**	**13**		**1.9**	**1.2**	**1.3**	**.94**	**.16**	**0**	**1.1**	**1.4**	**.59**	**2.7**	**2**	**2.7**	**2**	**2.0**	**2.0**				**3.7**	**0**	**.63**	**.3**	**.32**	**.111**	**.16**	**.75**	**1.2**	**.11**	**.37**	**.26**
			±SE	.033		.42			.13	.25	.09	.04	.09	0	.31	.23	.19	.33	.26	.67		.17	.11				.33	0		.18	.17	.111	.09	.19	.45	.07	.11	
PC	1	8	N	8		8	0		8	1	8	8	8	8	1	1	8		1			8	3				1	8	8	7	7	4	7	7	7	7	7	8
			mean	**.672**		**4.91**			**2.1**	**4**	**1.4**	**1**	**.63**	**0**	**2**	**2**			**2**			**1.6**	**1**				**3**	**0**	**.88**	**0**	**0**	**0**	**0**	**.29**	**.86**	**0**	**.86**	**0**
			±SE	.047		1.53			.13		.18	0	.32	0								.18	0					0		0	0	0	0	.18	.40	0	.40	
FA	3	6	N	6		6	0		6	5	6	6	6	6		4	6					6	6			1		6	6	2	2	1	2	2	2	2	2	6
			mean	**.447**		**4.95**			**2.2**	**1.2**	**1**	**1**	**0**	**0**		**1.3**	**.83**					**1.7**	**1.3**			**2**		**0**	**.33**	**0**	**.5**	**0**	**0**	**.5**	**.5**	**0**	**0**	**.33**
			±SE	.044		1.05			.31	.58	0	0	0	0		.25	.54					.21	.21					0		0	.5		0	.5	.5	0	0	
PC	3	8	N	8		8	1		8	8	8	8	8	8	1	2	8	1	1	1		8	7				1	8	8	7	7	4	7	7	7	7	7	8
			mean	**.609**		**5.50**	**13**		**2**	**2.3**	**1.1**	**1**	**.38**	**.25**	**2**	**1.5**	**.63**	**2**	**3**	**1**		**1.8**	**1.3**				**3**	**0**	**.88**	**.14**	**.29**	**1**	**0**	**.71**	**1.1**	**.14**	**2**	**.25**
			±SE	.031		.94			.19	.62	.13	0	.26	.25		.5	.42					.25	.18					0		.14	.29	.408	0	.36	.40	.14	.93	

DATA FOR GRAPHS (9-Mo samples):

			AFB1 Concentration (ppm)			
	Statistic	Diet	0	0.3	1.0	3
Weight (g)	N		74	27	32	6
	mean	**FA**	**.508**	**.526**	**.53**	**.45**
	±SE		.017	.035	.03	.04
	N		9	16	8	8
	mean	**PC**	**.691**	**.693**	**.67**	**.61**
	±SE		.077	.051	.05	.03
HG	N		74	26	32	6
	mean	**FA**	**2.1**	**2.0**	**1.9**	**2.2**
	±SE		.10	.17	.13	.31
	N		9	16	8	8
	mean	**PC**	**2.4**	**2.6**	**2.1**	**2**
	±SE		.29	.16	.13	.19
VE	N		73	27	18	5
	mean	**FA**	**1.5**	**1.4**	**1.2**	**1.2**
	±SE		.12	.18	.25	.58
	N		9	16	1	8
	mean	**PC**	**1.6**	**1.7**	**4**	**2.3**
	±SE		.41	.33		.62
DM	N		74	27	32	6
	freq.	FA	.66	.15	.09	0
	mean		**1.3**	**.4**	**.16**	**0**
	±SE		.12	.18	.09	0
	N		9	16	8	8
	freq.	PC	.56	.56	.38	.25
	mean		**1.4**	**1.1**	**.63**	**.38**
	±SE		.50	.30	.32	.26
APH	N		61	14	12	4
	mean	**FA**	**1.5**	**1.2**	**1.4**	**1.3**
	±SE		.09	.21	.23	.25
	N		6	10	1	2
	mean	**PC**	**2.2**	**1.8**	**2**	**1.5**
	±SE		.31	.13		.5
	N		74	26	32	6
	freq.		.45	.42	.25	.33

DATA FOR GRAPHS (9-Mo samples):

	Statistic	Diet	AFB1 Concentration (ppm) 0	0.3	1.0	3	
	mean	**FA**	**.9**	**1.0**	**.59**	**.83**	
SH	±SE		.13	.25	.19	.54	
	N		8	16	8	8	
	freq.			.44	0	.25	
	mean	**PC**		**.8**		**.63**	
	±SE			.23		.42	
	N		74	26	32	6	
	mean	**FA**	**1.4**	**2.0**	**2.0**	**1.7**	
HMA	±SE		.09	.14	.17	.21	
	N		8	16	8	8	
	mean	**PC**	**1.5**	**1.9**	**1.6**	**1.8**	
	±SE		.33	.14	.18	.25	
FCA	N	FA	73	26	32	6	
	freq.		.25	.7	.63	.33	
	N	PC	9	16	8	8	
	freq.		.22	.4	.88	.88	
NEO	N	FA	73	26	31	6	
	freq.		.01	.2	.26	.33	
	N	PC	9	16	8	8	
	freq.		0	.1	0	.25	
							Totals
SEX	M	FA	34	13	15	6	68
	F		38	9	9	0	56
	NP		3	5	8	0	16
	M	PC	2	6	2	6	16
	F		7	9	1	2	19
	NP		0	1	5	0	6